

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. §371		Attorney Docket Number 9150-0009.10
		U.S. Application No. (if known, see 37 CFR §1.5) 09/857651
International Application No. PCT/JP99/06881	International Filing Date 08 December 1999	Priority Date Claimed 09 December 1998
Title of Invention A METHOD FOR MANUFACTURING GLYCOPROTEINS HAVING HUMAN-TYPE GLYCOSYLATION		
Applicant(s) for DO/EO/US Tatsuji Seki, Kazuhito Fujiyama, Toshiomi Yoshida		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information		
1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. §371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. §371. 3. <input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. §371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. §371(b) and PCT Articles 22 and 39(1). 4. <input type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. §371(c)(2)) a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. §371(c)(2)). 7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. §371(c)(3)) a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. §371(c)(3)). 9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. §371(c)(4)) (unsigned). 10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. §371(c)(5)). Items 11. to 16. below concern document(s) or information included: 11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR §1.97 and §1.98. 12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR §3.28 and §3.31 is included. 13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSTITUTE preliminary amendment. 14. <input type="checkbox"/> A substitute specification. 15. <input type="checkbox"/> A change of Power of Attorney and/or Address letter. 16. <input checked="" type="checkbox"/> Other items or information: Certificate of Express Mail		

U.S. Application No. (if known, see 37 CFR \$1.5) <div style="font-size: 1.5em; font-weight: bold; text-align: center;">09/857651</div>	International Application No. <div style="font-weight: bold; text-align: center;">PCT/JP99/06881</div>	Attorney's Docket No. <div style="font-weight: bold; text-align: center;">9150-0009.10</div>			
17. <input checked="" type="checkbox"/> The following fees are submitted: BASIC NATIONAL FEE (37 CFR \$1.492(a)(1)-(5)): Search Report has been prepared by the EPO or JPO \$ 840.00 International preliminary examination fee paid to USPTO (37 CFR \$1.482) \$ 670.00 No international preliminary examination fee paid to USPTO (37 CFR \$1.482) but international search fee paid to USPTO (37 CFR \$1.445(a)(2)) \$ 760.00 Neither international preliminary examination fee (37 CFR \$1.482) nor international search fee (37 CFR \$1.445(a)(2)) paid to USPTO \$970.00 International preliminary examination fee paid to USPTO (37 CFR \$1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) \$ 96.00 <div style="text-align: right; font-weight: bold;">ENTER APPROPRIATE BASIC FEE AMOUNT =</div>		<div style="text-align: right; font-weight: bold;">CALCULATIONS</div> <div style="text-align: right; font-weight: bold;">PTO USE ONLY</div>			
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claim priority date (37 CFR \$1.492(e)).		<div style="text-align: right;">\$</div>			
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	14 - 20 =	0	0 x \$ 18.00	\$ 00.00	
Independent Claims	3 - 3 =	0	0 x \$ 78.00	\$ 00.00	
Multiple Dependent Claim(s) (if applicable)			+	\$	
TOTAL OF ABOVE CALCULATIONS =				\$840.00	
Reduction of % for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR \$1.9, \$1.27, \$1.28)				\$	
SUBTOTAL =				\$	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR \$1.942(f)).				\$	
Fee for recording the enclosed assignment (37 CFR \$1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR \$3.28, \$3.31). \$40.00 per property.				\$	
TOTAL FEES ENCLOSED =				\$840.00	
				Amount to be:	
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a. <input checked="" type="checkbox"/> A check in the amount of \$840.00 to cover the filing fee is enclosed. b. <input type="checkbox"/> Please charge Deposit Account No. <u>04-0531</u> in the amount of \$ <u> </u> to cover the above fees. This sheet is provided in triplicate. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>04-0531</u> . This sheet is provided in triplicate. <p style="font-size: 0.8em;">NOTE: Where an appropriate time limit under 37 CFR \$1.494 or \$1.495 has not been met, a petition to revive (37 CFR \$1.137(a) or (b)) must be filed and granted to restore the application to pending status.</p> <div style="display: flex; justify-content: space-between; align-items: flex-end;"> <div style="width: 40%;"> SEND ALL CORRESPONDENCE TO: Iota Pi Law Group P.O. Box 60850 Palo Alto, CA 94306 Customer No. 22918 </div> <div style="width: 50%; text-align: center;"> <div style="font-size: 1.2em; font-family: cursive;">Peter J. Dehlinger</div> <div style="font-size: 0.8em; margin-top: 5px;">Signature</div> <div style="margin-top: 10px;"> Name: <u>Peter J. Dehlinger</u> Registration No.: <u>28,006</u> </div> </div> </div>					

Rec'd PCT/PTO 27 AUG 2001

Attorney Docket No. 9150-0009.10

09/857651

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By: Lynne B. Anderson

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF:

Seki, et al.

SERIAL No.: 09/857,651

FILED: December 8, 1999

FOR: A METHOD FOR MANUFACTURING
GLYCOPROTEINS HAVING HUMAN-TYPE
GLYCOSYLATION

EXAMINER: Unknown

ART UNIT: Unknown

Preliminary Amendment

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examination, applicants respectfully request entry of the following amendments.

In the specification:

In the specification please replace the Sequence Listing on pages 1/8 through 8/8 with the Sequence Listing on pages 1-4 enclosed herewith.

09/857651

09/857651

Attorney Docket No. 9150-0009.10

531 Rec'd PC

07 JUN 2001

I hereby certify that this Preliminary Amendment is being filed with the following documents: application transmittal under 35 USC § 371, an oath or declaration of the inventors under 35 U.S.C. § 371, and an attached check, all being deposited with the United States Postal Service "Express Mail Post office to Addressee" service under 37 C.F.R. §1.10 on the date indicated below, and is addressed to the Assistant Commissioner for Patents, Box PCT, Washington, D.C. 20231, Attention: DO/EO/US.

Express Mail Label No. EL 530 372 429 US

Date: 6/7/01

By: Deborah H. Brockmeyer
Deborah H. Brockmeyer

DOCKET NO.: 9150-0009.10

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF:

Seki et al.

EXAMINER: unknown

SERIAL NO.: unknown

ART UNIT: unknown

FILED: concurrently herewith

FOR: A METHOD FOR MANUFACTURING
GLYCOPROTEINS HAVING HUMAN-TYPE
GLYCOSYLATION

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Before examination of the above-noted application, please amend the application as follows.

In the specification: On page 1, before line 1, insert the following paragraph:

This application claims priority to application no. PCT/JP99/06881 filed 08 December 1999, now publication no. WO 00/34490 published 15 June 2000; which claims priority to JP 10-350584 filed 09 December 1998, which are both incorporated herein by reference.

In the Claims: Please amend claims 2 – 7, 9, 10, and 13 with the following rewritten claims.

2. (Once Amended) The method according to claim 1, wherein the glycosyltransferase is an enzyme capable of conducting a transfer reaction of a galactose residue to a non-reducing

terminal acetylglucosamine residue.

3. (Once Amended) The method according to claim 1, wherein the glycoprotein with a human-type sugar chain comprises a core sugar chain and an outer sugar chain, wherein the core sugar chain comprises a plurality of mannose and acetylglucosamine, and wherein the outer sugar chain contains a terminal sugar chain portion with a non-reducing terminal galactose.
4. (Once Amended) The method according to claim 3, wherein the outer sugar chain has a straight chain configuration.
5. (Once Amended) The method according to claim 3, wherein the outer sugar chain has a branched configuration.
6. (Once Amended) The method according to claim 5, wherein the branched sugar chain portion has a mono-, bi-, tri-, or tetra configuration.
7. (Once Amended) The method according to claim 1, wherein the glycoprotein contains neither fucose nor xylose.
9. (Once Amended) The plant cell according to claim 8, wherein the plant cell is transformed with the gene of a first enzyme capable of conducting a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue and the gene of a second enzyme which can enhance the first enzyme.
10. (Once Amended) A plant cell according to claim 9, wherein the second enzyme is selected from the group consisting of Mannosidase I, Mannosidase II, β 1,4-Galactosyltransferase (GalT) and N-acetylglycosaminyltransferase I (GlcNAcT).
13. (Once Amended) A glycoprotein with a human-type sugar chain obtained using the method according to claim 1.

Attorney Docket No. 9150-0009.10

REMARKS

The claims have been amended to correct minor language and remove multiple dependencies. Entry of the amendments prior to examination is respectfully requested.

The Examiner is invited to contact Applicants' representative at 650-324-0880 if prosecution of this application would be assisted thereby.

Respectfully submitted,



Peter J. Dehlinger
Registration No. 28,006

Date:

6-7-2001**Correspondence Address:**

Customer No. 22918

097 857651

Attorney Docket No. 9150-0009.10

531 Rec'd PC

07 JUN 2001

VERSION WITH MARKINGS TO SHOW CHANGES MADE

2. (Once Amended) [A] The method according to claim 1, wherein the glycosyltransferase is an enzyme capable of conducting a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue.
3. (Once Amended) [A] The method according to [C]claim 1, wherein the glycoprotein with a human-type sugar chain comprises a core sugar chain and an outer sugar chain, wherein the core sugar chain comprises a plurality of mannose and acetylglucosamine, and wherein the outer sugar chain contains a terminal sugar chain portion with a non-reducing terminal galactose.
4. (Once Amended) [A] The method according to [C]claim 3, wherein the outer sugar chain has a straight chain configuration.
5. (Once Amended) [A] The method according to [C]claim 3, wherein the outer sugar chain has a branched configuration.
6. (Once Amended) [A] The method according to [C]claim 5, wherein the branched sugar chain portion has a mono-, bi-, tri-, or tetra configuration.
7. (Once Amended) [A] The method according to [C]claim 1 [through Claim 6], wherein the glycoprotein contains neither fucose nor xylose.
9. (Once Amended) [A] The plant cell according to claim 8, wherein the plant cell is transformed with the gene of a first enzyme capable of conducting a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue and the gene of a second enzyme which can enhance the first enzyme.
10. (Once Amended) [A] The plant cell according to claim 9, wherein the second enzyme is selected from the group consisting of Mannosidase I, Mannosidase II, β 1,4-Galactosyltransferase (GalT) and N-acetylglycosaminyltransferase I (GlcNAcT).

13. (Once Amended) A glycoprotein with a human-type sugar chain obtained using the method according to [C]claim 1 [through Claim 7].

DESCRIPTION

A METHOD FOR MANUFACTURING GLYCOPROTEINS HAVING HUMAN-TYPE GLYCOSYLATIONTECHNICAL FIELD

- 5 The present invention relates to expression of exogenous glycoproteins by plants.

BACKGROUND ART

- 10 Many of the functional proteins in living organisms are glycoproteins. It has been elucidated that the diversity of the sugar chains in glycoproteins play several important roles physiologically (Lain, R.A., Glycobiology, 4, 759-767, 1994).
- 15 In recent years, it has also become clear that the action of sugar chains can be divided into two categories. In the first case, sugar chains have a direct function as ligands for binding cells, or as receptors for bacteria and viruses, in the clearance of glycoproteins from the blood, lysosome
- 20 targeting of lysosome enzymes and the targeting by glycoproteins toward specific tissues and organs. For example, the contribution of glycoprotein sugar chains in the infection of target cells by the AIDS virus (HIV) has been established (Rahebi, L. et al., Glycoconj. J., 12, 7-16,
- 25 1995). The surface of HIV is covered with envelope protein gp120. The binding of gp120 sugar chains to the CD4 of target cells is the beginning of infection by the HIV virus. In the second case, the sugar chain itself is not the functional molecule but indirectly contributes to the formation of the
- 30 higher-order structure of proteins, solubility of proteins, protease resistance of proteins, inhibition of antigenicity, protein function modification, protein regeneration rate adjustment, and adjustment of the amount of proteins

expressed in cell layers. For example, sugar chains are instrumental in the adjustment of the adhesion of nerve cell adhesion molecules which are distributed widely in the nervous system (Edelman, G.M., Ann. Rev. Biochem., 54, 135-169, 1985).

In eukaryotes, glycoprotein sugar chains are synthesized on lipids of the Endoplasmic reticulum as precursor sugar chains. The sugar chain portion is transferred to the protein, then some of the sugar residues on the protein are removed in the Endoplasmic reticulum, and then the glycoprotein is transported to Golgi bodies. In the Golgi bodies, after the excess sugar residues have been removed, further sugar residues (e.g. mannose) are added and the sugar chain is extended (Narimatsu, H., Microbiol. Immunol., 38, 489-504, 1994).

More specifically, for example, Glc3Man9GlcNAc2 on dolichol anchors is transferred to protein in the ER membrane (Moremen K.W., Trimble, R.B. and Herscovics A., *Glycobiology* 1994 Apr;4(2):113-25, Glycosidases of the asparagine-linked oligosaccharide processing pathway; and Sturm, A. 1995 N-Glycosylation of plant proteins. In: *New Comprehensive Biochemistry. Glycoproteins*, Vol.29a., Montreuil, J., Schachter, H. and Vliegenthart, J.F.G.(eds). Elsevier Science Publishers B.V., The Netherland, pp. 521-541). ER-glucosidase I and II removes three glucose units (Sturm, A. 1995, *supra*; and Kaushal G.P. and Elbein A.D., 1989, Glycoprotein processing enzymes in plants. In *Methods Enzymology* 179, Complex Carbohydrates Part F. Ginsburg V. (ed), Academic Press, Inc. NY, pp.452-475). The resulting high mannose structure (Man9GlcNAc2) is trimmed by ER-mannosidase (Moremen K.W. et al, *supra*; and Kornfeld, R.

and Kornfeld, S., *Annu. Rev. Biochem.* 54, 631-664, 1985; Assembly of asparagine-linked oligosaccharides). The number of mannose residues removed varies according to the differences in the accessibility to the processing enzymes.

5 The isomers Man8-, Man7-, Man6- and Man5GlcNAc2 are produced during processing by ER-mannosidase and Mannosidase I (Kornfeld, R. and Kornfeld, S., *supra*). When four mannose residues are completely removed by Mannosidase I (Man I), the product is Man5GlcNAc2. N-acetylglucosaminyl

10 transferase I (GlcNAc I) transfers N-acetylglucosamine (GlcNAc) from UDP-GlcNAc to Man5GlcNAc2, resulting in GlcNAcMan5GlcNAc2 (Schachter, H., Narasimhan, S., Gleeson, P., and Vella, G., *Glycosyltransferases involved in elongation of N-glycosidically linked oligosaccharides of the complex or N-acetylgalactosamine type. In: Methods Enzymol* 98: Biomembranes Part L. Fleischer, S., and Fleischer, B. (ed), Academic Press, Inc. NY, pp.98-134 pp. 98-134, 1983). Mannosidase II (Man II) removes two mannose

15 residues from GlcNAcMan5GlcNAc2, yielding GlcNAcMan3GlcNAc2 (Kaushal, G.P. and Elbein, A.D., *supra*; and Kornfeld, R. and Kornfeld, S., *supra*). The oligosaccharide GlcNAcMan4GlcNAc2 is used as a substrate of N-acetylglucosaminyl transferase II (GlcNAc II) (Moremen K.W. et al, *supra*; Kaushal, G.P. and Elbein, A.D., *supra*;

25 and Kornfeld, R. and Kornfeld, S., *supra*). FIG 19 summarizes the above described structures of N-linked glycans and enzymes involved in sugar chain modification pathway in the Endoplasmic reticulum and Golgi bodies. In FIG 19, \diamond denotes glucose, \square denotes GlcNAc, \circ denotes mannose, \bullet denotes galactose, and \blacksquare denotes sialic acid, respectively.

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The sugar addition in the Golgi bodies is called terminal sugar chain synthesis. The process differs widely among

living organisms. The sugar chain synthesis depends on the type of eukaryote. The resulting sugar chain structure is species-specific, and reflects the evolution of sugar adding transferase and the Golgi bodies (Narimatsu, H., Cellular Biology, 15, 802-810, 1996).

Regarding asparagine-linked (N-linked) sugar chains; in animals, there are high mannose-type sugar chains, complex-type sugar chains and hybrid-type sugar chains. These structures are shown in FIG 1. The complex-type sugar chains in plants have α 1,3 fucose and β 1,2 xylose which are sugar residues that are not found in animals (Johnson, K.D. and Chrispeels, M.J., Plant Physiol., 84, 1301-1308, 1897, Kimura, Y. et al., Biosci. Biotech. Biochem., 56, 215-222, 1992). In the case of N-linked sugar chains, sialic acid has been found in animal sugar chains but has not been found in plant sugar chains. Regarding galactose, which is generally found in animal sugar chains, although the presence thereof has been found in some plant sugar chains (Takahashi, N. and Hotta, T., Biochemistry, 25, 388-395, 1986), the examples thereof are few. The linkage-type thereof is a β 1,3 linkage (FEBS Lett 1997 Sep 29, 415(2), 186-191, Identification of the human Lewis(a) carbohydrate motif in a secretory peroxidase from a plant cell suspension culture (*Vaccinium mytillus* L.), Melo NS, Nimtz M, Contradt HS, Fevereiro PS, Costa J; Plant J. 1997 Dec. 12(6), 1411-1417, N-glycans harboring the Lewis a epitope are expressed at the surface of plant cells., Fitchette-Laine AC, Gomord V, Cabanes M, Michalski JC, Saint Macary M, Foucher B, Cavelier B, Hawes C, Lerouge P, Faye L). This linkage is different from those found in animals.

Glycoproteins derived from humans include human

erythropoietin (EPO). In order to produce glycoproteins with sugar chain structures similar to humans, these glycoproteins are produced in animal host cells. However, EPO produced in animal cells has a sugar chain structure that is different from the natural human sugar chain structure. As a result, in vivo activity of EPO is reduced (Takeuchi, M. et al., Proc. Natl. Acad. Sci. USA, 86, 7819-7822, 1989). The sugar chain structure in other proteins derived from humans, such as hormones and interferon, have also been analyzed and manufactured with the same glycosylation limitations.

The methods used to introduce exogenous genes to plants include the *Agrobacterium* method (Weising, K. et al., Annu. Rev. Genet., 22, 421, 1988), the electroporation method (Toriyama, K. et al., Bio/Technology, 6, 1072, 1988), and the gold particle method (Gasser, C.G. and Fraley, R.T., Science, 244, 1293, 1989). Albumin (Sijmons, P.C. et al., Bio/Technology, 8, 217, 1990), enkephalin (Vandekerckhove, J. et al., Bio/Technology, 7, 929, 1989), and monoclonal antibodies (Benvenuto, E. et al., Plant Mol. Biol., 17, 865, 1991 and Hiatt, A. et al., Nature, 342, 76, 1989) have been manufactured in plants. Hepatitis B virus surface antigens (HBsAg) (Mason, H.S. et al., Proc. Natl. Acad. Sci. USA., 89, 11745, 1992) and secretion-type IgA (Hiatt, A. and Ma, J.S.K., FEBS Lett., 307, 71, 1992) have also been manufactured in plant cells. However, when human-derived glycoproteins are expressed in plants, the sugar chains in the manufactured glycoproteins have different structures than the sugar chains in the glycoproteins produced in humans because the sugar adding mechanism in plants is different from the sugar adding mechanism in animals. As a result, glycoproteins do not have the original physiological

activity and may be immunogenic in humans (Wilson, I.B.H. et al., *Glycobiol.*, Vol. 8, No. 7, pp. 651-661, 1998).

DISCLOSURE OF THE INVENTION

- 5 The purpose of the present invention is to solve the problems associated with the prior art by providing plant-produced recombinant glycoproteins with mammalian, e.g., human-type sugar chains.
- 10 The present invention is a method of manufacturing a glycoprotein having a human-type sugar chain comprising a step in which a transformed plant cell is obtained by introducing to a plant cell the gene of an enzyme capable of conducting a transfer reaction of a galactose residue
- 15 to a non-reducing terminal acetylglucosamine residue and the gene of a exogenous glycoprotein, and a step in which the obtained transformed plant cell is cultivated.
- 20 In the present invention, the glycoprotein with a human-type sugar chain can comprise a core sugar chain and an outer sugar chain, the core sugar chain consists essentially of a plurality of mannose and acetylglucosamine, and the outer sugar chain contains a terminal sugar chain portion with a non-reducing terminal galactose.
- 25 In the present invention, the outer sugar chain can have a straight chain configuration or a branched configuration. In the present invention, the branched sugar chain portion can have a mono-, bi-, tri- or tetra configuration.
- 30 In the present invention, the glycoprotein can contain neither fucose nor xylose.

The present invention is also a plant cell having a sugar

chain adding mechanism which can conduct a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue, wherein the sugar chain adding mechanism acts on a sugar chain containing a core sugar chain and an outer sugar chain, wherein the core sugar chain consists essentially of a plurality of mannose and acetylglucosamine, and wherein the outer sugar chain contains a terminal sugar chain portion with a non-reducing terminal galactose.

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In the present invention, a glycoprotein with a human-type sugar chain is obtained using this method.

BRIEF DESCRIPTION OF DRAWINGS

15 FIG 1. A schematic drawing of typical N-linked sugar chain configurations.

FIG 2. Schematic drawings of the cloning method for hGT.

FIG 3. Schematic drawings of the method used to construct vector pGAhGT for hGT expression.

20 FIG 4. A photograph showing a Southern blot analysis of a genome of cultivated transformed tobacco cells. FIG 4 (A) shows electrophoresis after the genome DNA (40 μ g) has been digested by EcoRI and HindIII. The numbers at the left indicate the position of the DNA molecular weight marker.

25 FIG 4 (B) shows a schematic drawing of a 2.2 kb fragment containing a promoter, hGT and terminator, which is integrated into the transformed cell.

FIG 5. FIG 5 is a photograph of the Western blotting of immunoreactive protein from transformed tobacco BY2 cells (WT) and wild type tobacco BY2 cells (WT). The protein was denatured, electrophoresed on 10% SDS-PAGE, and then transferred electrically to nitrocellulose film. The samples were as follows: lane 1 = GT1 cell extract; lane

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2 = GT 6 cell extract; lane 3 = GT8 cell extract; lane 4 = GT9 cell extract; lane 5 = wild type cell extract; lane 6 = GT1 microsome fragment; lane 7 = GT6 microsome fragment; lane 8 = GT8 microsome fragment; lane 9 = GT9 microsome fragment; lane 10 = wild type microsome fragment.

FIG 6. An electrophoresis photograph showing the detection of galactosylated glycoprotein using *Ricinus communis* (RCA₁₂₀) affinity chromatography. The electrophoresed gel was visualized by silver staining. Lanes 1 and 2 show the protein from wild type BY2 cells, while Lanes 3 and 4 show the protein from transformed GT6 cells. The molecular weight is in KDa units.

FIG 7. A photograph of Western blotting a showing the detection of galactosylated glycoprotein using *Ricinus communis* (RCA₁₂₀) affinity chromatography. After the electrophoresed gel had been blotted on a nitrocellulose membrane, this membrane was visualized by lectin (RCA₁₂₀) staining. Lanes 1 and 2 show the protein from a wild type BY2 cell, while Lanes 3 and 4 show the protein from transformed GT6 cells. The molecular weight is in KDa.

FIG 8. A photograph of a blotting in which the galactosylated glycoprotein from *Ricinus communis* (RCA₁₂₀) affinity chromatography was probed with an antiserum specific to xylose in complex-type plant glycans. Lanes 1 and 2 show the total protein extracts from BY2 and GT6, respectively, and Lane 3 shows the glycoprotein from GT6 after RCA₁₂₀ affinity chromatography. The molecular weight is in KDa units.

FIG 9. A schematic drawing of a plasmid pBIHm-HRP which is a binary vector with a kanamycin-resistant gene and a hygromycin-resistant gene, and has a HRP cDNA.

FIG 10. Photographs of isoelectric focusing and Western blotting which show HRP production in a suspension culture

of transgenic cells. FIG 10 (A) shows the results of isoelectric focusing and FIG 10 (B) shows the results of Western blotting. The abbreviations are as follows: WT = wild-type; BY2 - HRP 1, 5 and 7 = the clone numbers for BY2 cells transformed with a HRP gene; and GT-6 - HRP 4, 5 and 8 = the clone numbers for GT6 cells transformed with a HRP gene.

FIG 11. A graph showing the reverse-phase HPLC pattern of a PA sugar chain eluted in 0-15% acetonitrile linear gradient in 0.02% TFA over 60 minutes and at a flow rate of 1.2 ml/min. I-XI shows the fractions eluted and purified from size-fractionation HPLC. Excitation wavelength and emission wavelength were 310nm and 380nm, respectively.

FIG 12. Graphs showing the size-fractionation HPLC pattern of the PA sugar chain in FIG 11. Elution was performed in a 30-50% water gradient in the water-acetonitrile mixture over 40 minutes and at a flow rate of 0.8 ml/min. The excitation wavelength and emission wavelength were 310 nm and 380 nm, respectively.

FIG 13. A graph showing the elution position of peak-K2 on reverse phase HPLC wherein two standard sugar chain products A and B are compared with the peak K2. The elution conditions were the same as in FIG 11. That is, elution was performed in 0-15% acetonitrile linear gradient in 0.02% TFA over 60 minutes and at a flow rate of 1.2 ml/min.

FIG 14. Graphs showing the SF-HPLC profiles of galactosylated PA sugar chains obtained after exoglycosidase digestion. Elution was performed in a 30-50% water gradient in the water-acetonitrile mixture over 25 minutes and at a flow rate of 0.8 ml/min. (A) PA-sugar chain K-2: I is the elution position of the galactosylated PA sugar chain used; II is β -galactosidase digests of I; III is a N-acetyl- β -D-glucosaminidase digests of II; IV is

jack bean α -mannosidase digests of III. (B) PA-sugar chain L: I is the elution position of the galactosylated PA sugar chain used; II is β -galactosidase digests of I; III is N-acetyl- β -D-glucosaminidase digests of II; IV is α 1,2
5 mannosidase digests of III; V is jack bean α -mannosidase digests of III.

FIG 15. Estimated structures of the N-linked glycans obtained from the transformed cells. The numbers in the parentheses indicate the molar ratio.

10 FIG 16. Photographs of *Ricinus communis* 120 agglutinin (RCA₁₂₀) affinity chromatography showing the detection of glycosylated HRP. FIG 16 (A) shows the results from silver staining, and FIG 16 (B) shows the results from lectin RCA₁₂₀ staining. The lectin-stained filter was cut into strips and
15 then probed using lectin RCA₁₂₀ pre-incubated with buffer alone (I and II) or incubated in buffer with excess galactose (III). In (II), HRP was treated with β -galactosidase from *Diplococcus pneumoniae* before SDS-PAGE. Lane 1 is a collected fraction containing BY2-HRP and Lane 2 is a
20 collected fraction containing GT6-HRP. The numbers to the left refer to the location and the size (KDa) of the standard protein.

FIG 17. A graph showing the results of reverse-phase HPLC of the PA sugar chains from purified HRP after RCA₁₂₀ affinity chromatography.
25

FIG 18. Photographs of Western blotting showing immune detection of plant specific complex-type glycans. The purified HRP is fractionated by SDS-PAGE, transferred to nitrocellulose, and confirmed with rabbit anti-HRP (A) and
30 an antiserum which is specific for complex-type glycans of plants (B). Lane 1 = galactosylated HRP from GT6-HRP after RCA₁₂₀ affinity chromatography; Lane 2 = purified HRP from BY2-HRP. The position of the molecule size marker is shown

to the left in KDa. The galactosylated N-glycan on HRP derived from the transformant GT6-HRP cells did not react with an antiserum which has been shown to specifically react with β 1,2 xylose residue indicative of plant N-glycans.

5 FIG 19. Structures of N-linked glycans and enzymes involved in the sugar chain modification pathway in Endoplasmic reticulum and Goldi bodies. \diamond denotes glucose, \square denotes GlcNAc, \bigcirc denotes mannose, \bullet denotes galactose, and \blacksquare denotes sialic acid, respectively.

10 FIG 20. Structures of N-linked glycans and the ratio of each N-linked glycan in GT6 cell line along with those in wild-type BY2 cell line determined similarly. \square denotes GlcNAc, \bigcirc denotes mannose, \bullet denotes galactose, and \blacksquare denotes sialic acid, respectively.

15 FIG 21 illustrates one of the embodiment of the present invention. In GT6 cell line, the isomers Man7-, Man6- and Man5GlcNAc2 were observed. Because those high-mannose type oligosaccharides will be converted by some glycan processing enzymes to be substrates for β 1,4-galactosyltransferase (Gal T), introduction of GlcNAc I, Man I and Man II cDNAs
20 could more efficiently lead the oligosaccharide Man7-5GlcNAc2 to GlcNAcMan3GlcNAc2, which can be a substrate of GalT.

FIG 22 also illustrates another the embodiment of the present invention. 1,4-Galactosyltransferase (Gal T) uses UDP-galactose as a donor substrate and GlcNAc2Man3GlcNAc2 as an acceptor substrate. Efficient supply of UDP-galactose will enhance the Gal T enzyme reaction and more galactosylated oligosaccharide will be produced.

30

BEST MODE FOR CARRYING OUT THE INVENTION

Hereinafter, the present invention will be described in further detail. In performing the present invention,

unless otherwise indicated, any conventional technique can be used. These include methods for isolating and analyzing proteins as well as immunological methods. These methods can be conducted by using commercial kits, antibodies and markers.

The method of the present invention relates to a method of manufacturing glycoproteins with human-type sugar chains. In this specification, "human-type sugar chain" refers to a sugar chain with a galactose residue linked to a N-acetylglucosamine residue. The galactose residue in the human-type sugar chain can be the terminal sugar chain or a sialic acid residue can be linked to the outside of the galactose residue. Preferably, the glycoprotein of the present invention at least has no xylose or fucose linked to one or more of the following portions: the core sugar chain portion, the branched sugar chain portion, or the terminal sugar chain portion of the human-type sugar chain. More preferably, neither xylose or fucose should be linked to any portion of the human-type sugar chain, and ideally there should be no xylose or fucose contained in the human-type sugar chain at all.

The plant cells can be any plant cells desired. The plant cells can be cultured cells, cells in cultured tissue or cultured organs, or cells in a plant. Preferably, the plant cells should be cultured cells, or cells in cultured tissue or cultured organs. Most preferably, the plant cells should be cells in whole plants, or portions thereof, that produce glycoproteins with human-type sugar chains. The type of plant used in the manufacturing method of the present invention can be any type of plant that is used in gene transference. Examples of types of plants that can be used

in the manufacturing method of the present invention include plants in the families of *Solanaceae*, *Poaceae*, *Brassicaceae*, *Rosaceae*, *Leguminosae*, *Curcubitaceae*, *Lamiaceae*, *Liliaceae*, *Chenopodiaceae* and *Umbelliferae*.

5

Examples of plants in the *Solanaceae* family include plants in the *Nicotiana*, *Solanum*, *Datura*, *Lycopersicon* and *Petunia* genera. Specific examples include tobacco, eggplant, potato, tomato, chili pepper, and petunia.

10

Examples of plants in the *Poaceae* family include plants in the *Oryza*, *Hordenum*, *Secale*, *Saccharum*, *Echinochloa* and *Zea* genera. Specific examples include rice, barley, rye, *Echinochloa crus-galli*, sorghum, and maize.

15

Examples of plants in the *Brassicaceae* family include plants in the *Raphanus*, *Brassica*, *Arabidopsis*, *Wasabia*, and *Capsella* genera. Specific examples include Japanese white radish, rapeseed, *Arabidopsis thaliana*, Japanese horseradish, and *Capsella bursa-pastoris*.

20

Examples of plants in the *Rosaceae* family include plants in the *Orunus*, *Malus*, *Pynus*, *Fragaria*, and *Rosa* genera. Specific examples include plum, peach, apple, pear, Dutch strawberry, and rose.

25

Examples of plants in the *Leguminosae* family include plants in the *Glycine*, *Vigna*, *Phaseolus*, *Pisum*, *Vicia*, *Arachis*, *Trifolium*, *Alfalfa*, and *Medicago* genera. Specific examples include soybean, adzuki bean, kidney beans, peas, fava beans, peanuts, clover, and alfalfa.

30

Examples of plants in the *Curcubitaceae* family include

plants in the *Luffa*, *Curcubita*, and *Cucumis* genera. Specific examples include gourd, pumpkin, cucumber, and melon.

- 5 Examples of plants in the *Lamiaceae* family include plants in the *Lavandula*, *Mentha*, and *Perilla* genera. Specific examples include lavender, peppermint, and beefsteak plant.

- 10 Examples of plants in the *Liliaceae* family include plants in the *Allium*, *Lilium*, and *Tulipa* genera. Specific examples include onion, garlic, lily, and tulip.

- 15 Examples of plants in the *Chenopodiaceae* family include plants in the *Spinacia* genera. A specific example is spinach.

- 20 Examples of plants in the *Umbelliferae* family include plants in the *Angelica*, *Daucus*, *Cryptotaenia*, and *Apitum* genera. Specific examples include Japanese udo, carrot, honewort, and celery.

- 25 Preferably, the plants used in the manufacturing method of the present invention should be tobacco, tomato, potato, rice, maize, radish, soybean, peas, alfalfa or spinach. Ideally, the plants used in the manufacturing method of the present invention should be tobacco, tomato, potato, maize or soybean.

- 30 In this specification, "an enzyme capable of conducting a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue" refers to an enzyme capable of conducting a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine

residue produced when a sugar chain is added after synthesis of the protein portion of the glycoprotein in the plant cell. Specific examples of these enzymes include galactosyltransferase, galactosidase, and β -galactosidase. 5 These enzymes can be derived from any animal desired. Preferably, these enzymes should be derived from a mammal, and ideally these enzymes should be derived from a human.

10 In this specification, "the gene of an enzyme capable of conducting a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue" can be a gene which can be isolated from an animal cell using a nucleotide sequence of an encoded enzyme well known in the art, or commercially available genes altered for expression 15 in plants.

In this specification, "gene" usually refers to the structural gene portion. A control sequence such as a promoter, operator and terminator can be linked to the gene 20 so as to properly express the gene in a plant.

In this specification, "exogenous glycoproteins" refers to glycoproteins whose expression in plants is the result of genetic engineering methodologies. Examples of these 25 exogenous glycoproteins include enzymes, hormones, cytokines, antibodies, vaccines, receptors and serum proteins. Examples of enzymes include horseradish peroxidase, kinase, glucocerebrosidase, α -galactosidase, tissue-type plasminogen activator (TPA), and HMG-CoA 30 reductase. Examples of hormones and cytokines include enkephalin, interferon alpha, GM-CSF, G-CSF, chorion stimulating hormone, interleukin-2, interferon-beta, interferon-gamma, erythropoietin, vascular endothelial

growth factor, human choriogonadotropin (HCG),
leuteinizing hormone (LH), thyroid stimulating hormone
(TSH), prolactin, and ovary stimulating hormone. Examples
of antibodies include IgG and scFv. Examples of vaccines
5 include antigens such as Hepatitis B surface antigen,
rotavirus antigen, Escherichia coli enterotoxin, malaria
antigen, rabies virus G protein, and HIV virus glycoprotein
(e.g., gp120). Examples of receptors and matrix proteins
include EGF receptors, fibronectin, α 1-antitrypsin, and
10 coagulation factor VIII. Examples of serum proteins
include albumin, complement proteins, plasminogen,
corticosteroid-binding globulin, thyroxine-binding
globulin, and protein C.

15 In this specification, "genes of exogeneous glycoproteins"
refers to a gene, which can be isolated from a cell using
a nucleotide sequence of an encoded protein well known in
the art, or commercially available genes altered for
expression in plants.

20 The gene of the enzymes capable of conducting a transfer
reaction of a galactose residue to a non-reducing terminal
acetylglucosamine residue and the genes of exogenous
glycoproteins can be introduced to the plant cells using
25 a method well known in the art. These genes can be introduced
separately or simultaneously. Examples of methods for
introducing genes to plant cells include the Agrobacterium
method, the electroporation method and the particle
bombardment method.

30 Using any method well known in the art, the plant cells with
introduced genes can be tested to make sure the introduced
genes are expressed. Examples of such methods include

silver staining or augmentation, Western blotting, Northern hybridization, and enzyme activity detection. Cells that express the introduced genes are referred to as transformed cells.

- 5 Transformed cells, which express enzymes capable of conducting a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue and exogenous glycoproteins, express exogenous glycoproteins with human-type sugar chains. In other words, the
- 10 transformed cells have human-type sugar chain adding mechanisms. By cultivating these transformed cells, glycoproteins with human-type sugar chains can be mass produced. Human-type glycoproteins contain core sugar chains and outside sugar chains. The core sugar chains
- 15 consist essentially of a plurality of mannose or acetylglucosamine. The outside sugar chains in these glycoproteins contain non-reducing terminal sugar chain portions. The outside sugar chains can have a straight chain configuration or a branched chain configuration. The
- 20 branched sugar chain portion has a mono-, bi-, tri- or tetra configuration. The glycoproteins manufactured using these transformed cells ideally do not contain any fucose or xylose.
- 25 These transformed plant cells can remain in a cultured state or can be differentiated into specific tissues or organs. Alternatively, they can also be generated into plants. In this case, the transformed plant cells can be present in the entire plant or in specific portions of the plant, such
- 30 as seed, fruit, nut, leaf, root, stem or flower of the plant.

Glycoproteins with human-type sugar chains can be manufactured by the transformed plant cells and then be

isolated or extracted from the plant cells. The method for isolating the glycoproteins can be any method well known in the art. The glycoproteins of the present invention can be used in foodstuffs while remaining inside the transformed
5 cells, or the glycoproteins of the present invention can be administered to animals including humans without antigenicity because of the added human-type sugar chains.

Hereinafter, the present invention will be described in
10 detail by way of illustrative, but not restrictive, examples.

(Example 1) Cloning Human β 1,4 Galactose Transferase Genes
 β 1,4 Galactosyltransferase (hGT) genes (EC2.4.1.38) have
15 already been cloned. A primary configuration consisting of 400 amino acids has been discovered (Masri, K.A. et al., Biochem. Biophys. Res. Commun., 157, 657-663, 1988).

(1) Primer Preparation and Template DNA

The following primers were prepared with reference to the
20 report by Masri et al.

hGT-5Eco: 5'-AAAGAATTCGCGATGCCAGGCGCGCTCCCT-3' (Sequence ID:1)

hGT-2Sal: 3'-TCGATCGCAAAACCATGTGCAGCTGATG-5' (Sequence I.D:2)

25 hGT-7Spe: 3'-ACGGGACTCCTCAGGGGCGATGATCATAA-5' (Sequence I.D:3)

hGT6Spe: 5'-AAGACTAGTGGGCCCCATGCTGATTGA-3' (Sequence I.D:4)

Human genome DNA, human placenta cDNA, and human kidney cDNA
30 purchased from Clontech were used as the template DNA.

(2) Cloning the hGT Gene cDNA

(i) Human genome DNA was used as the template and hGT-5Eco

and hGT-7Spe were used as the primers; (ii) Human placenta cDNA was used as the template and hGT-2Sal and hGT6Spe were used as the primers. The two were combined and a PCR reaction was performed under the following conditions. Then, 0.4 kb and 0.8 kb fragments containing hGT encoded areas were obtained.

(PCR reaction mixture) 1 μ l template DNA, 5 μ ml 10 x PCR buffer solution, 4 μ l dNTPs (200 mM), the primers (10 pmol), and 0.5 μ l (Takara Shuzo Co., Ltd.) Tag polymerase (or 0.2 μ l Tub polymerase), water was added to make 50 μ l.

(PCR Reaction Conditions) First Stage: 1 cycle, denaturation (94°C) 5 minutes, annealing (55°C) 1 minute, extension (72°C) 2 minutes. Second Stage: 30 cycles, denaturation (94°C) 1 minute, annealing (55°C) 1 minute, extension (72°C) 2 minutes. Third Stage: 1 cycle, denaturation (94°C) 1 minute, annealing (55°C) 2 minutes, extension (72°C) 5 minutes.

The two fragments were combined to form hGT gene cDNA and cloned in pBluescript II SK+ (SK). The pBluescript II SK+ (SK) was purchased from Stratagene Co., Ltd. FIG 2 shows the structure of a plasmid containing hGT gene cDNA. This shows Sequence No. 5 in the hGT gene nucleotide sequence and Sequence No. 6 in the estimated amino acid sequence. This nucleotide sequence differed from the hGT sequence published by Masri et al. (see above) in the following ways:

a) The nucleotides are different in that the A in Position No. 528 is G, the C in Position No. 562 is T, and the A in Position No. 1047 is G, however the encoded amino acid sequence is not changed; b) Nine nucleotides at positions from Position No. 622 to Position No. 630 are missing; c) The G in Position No. 405 is A and the T in Position No. 408 is A. These nucleotide changes were made during primer preparation such that the 0.4 kb fragment and 0.8 kb fragment

are connected. There are two start codons (ATG) in hGT gene cDNA. In this experiment, however, the gene is designed such that translation begins from the second initial codon (Position No. 37).

5

(Example 2) Introduction of the hGT Gene to a Cultivated Tobacco Cell

(1) It has been reported that hGT is expressed in an active form in *Escherichia coli* (Aoki, D. et al., EMBO J., 9, 3171, 1990 and Nakazawa, K. et al., J. Biochem., 113, 747, 1993). In order for a cultivated tobacco cell to express hGT, the expression vector pGAhGT had to be structured as shown in FIG 3. A cauliflower mosaic virus 35S promoter (CaMV 35S promoter), which drives gene expression constitutively in plant cells, was used as the promoter. A kanamycin-resistance gene was used as the selection marker. The pGAhGT was introduced to the cultivated tobacco cell by means of *Agrobacterium* method.

20 The *Agrobacterium* method was performed using the triparental mating method of Bevan et al. (Bevan, M., Nucleic Acid Res., 12, 8711, 1984). *Escherichia coli* DH5 α (suE44, DlacU169, (ϕ 80lacZDM15), hsdR17) (Bethesda Research Laboratories Inc.: Focus 8 (2), 9, 1986) with pGA-type plasmids (An. G., 25 Methods Enzymol. 153, 292, 1987) and *Escherichia coli* HB101 with helper plasmid pRK2013 (Bevan, M., Nucleic Acid Res., 12, 8711, 1984) were left standing overnight and 37°C in a 2 x YT medium containing 12.5 mg/l tetracycline and 50 mg/l kanamycin, and *Agrobacterium tumefaciens* EHA101 was left 30 standing over two nights at 28°C in a 2 x YT medium containing 50 mg/l kanamycin and 25 mg/l chloramphenicol. Then, 1.5 ml of each cultured medium was removed and placed into an Eppendorf tube. After the cells of each strain were

collected, the cells were rinsed three times in an LB medium. The cells obtained in this manner were then suspended in 100 μ l of a 2 x YT medium, mixed with three types of bacteria, applied to a 2 x YT agar medium, and cultivated at 28°C whereby
5 the pGA-type plasmids, then underwent conjugal transfer from the *E. coli* to the *Agrobacterium*. Two days later some of the colony appearing on the 2 x YT agar plate was removed using a platinum loop, and applied to an LB agar plate containing
10 50 mg/l kanamycin, 12.5 mg/l tetracycline, and 25 mg/l chloramphenicol. After cultivating the contents for two days at 28°C, a single colony was selected.

Transformation of the cultivated tobacco cells was performed using the method described in An, G., Plant Mol. Bio. Manual,
15 A3, 1. First, 100 μ l of *Agrobacterium* EHA101 with pGA-type plasmids cultivated for 36 hours at 28°C in an LB medium containing 12.5 mg/l tetracycline and 4 ml of a suspension of cultivated tobacco cells *Nicotiana tabacum* L. cv. bright
20 yellow 2 (Strain No. BY-2 obtained using Catalog No. RPC1 from the Plant Cell Development Group of the Gene Bank at the Life Science Tsukuba Research Center), in their fourth day of cultivation, were mixed together thoroughly in a dish and allowed to stand in a dark place at 25°C. Two days later,
25 some of the solution was removed from the dish and the supernatant was separated out using a centrifuge (1000 rpm, 5 minutes). The cell pellet was introduced to a new medium and centrifuged again. The cells were innoculated onto a modified LS agar plate with 150-200 mg/l kanamycin and 250
30 mg/l carbenicillin. This was allowed to stand in darkness at 25°C. After two to three weeks, the cells grown to the callus stage were transferred to a new plate and clones were selected. After two to three weeks, the clones were transferred to a 30 ml modified LS medium with kanamycin

and carbenicillin. This selection process was repeated over about one month. Six resistant strains were randomly selected from the resistant strains obtained in this manner (GT 1, 4, 5, 6, 8 and 9).

5

(2) Verification of the Introduced hGT Genes

In the resistant strains, a 2.2 kb fragment containing a CaMV35S promoter and an hGT gene cDNA-NOS terminator in the T-DNA was confirmed in the genomic DNA of the cultivated tobacco cells using a Southern blot analysis. The Southern method was performed after the genomic DNA had been prepared from the resistant strains mentioned above and digested by EcoRI and HindIII.

15 The preparation of the chromosomal DNA from the cultured tobacco cells was performed using the Watanabe method (Watanabe, K., Cloning and Sequence, Plant Biotechnology Experiment Manual, Nouseon Bunka Co., Ltd.). First, 10 ml of the cultivated tobacco cells were frozen using liquid
20 nitrogen, and then ground to powder using a mortar and pestle. About five grams of the powder was placed in a centrifuge tube (40 ml) rapidly such that the frozen powder did not melt and mixed with 5 ml of a 2 x CTAB (cetyltrimethyl ammonium bromide) solution pre-heated to 60°C. This was well mixed,
25 slowly for 10 minutes, and then allowed to stand at 60°C. Then, 5 ml of a chloroform:isoamylalcohol (24:1) solution was added, and the mixture was stirred into an emulsion. The mixture was then centrifuged (2,800 rpm, 15 minutes, room temperature). The surface layer was then transferred
30 to a new 40 ml centrifuge tube and the extraction process was repeated using the chloroform:isoamylalcohol (24:1) solution. After the surface layer had been mixed with 1/10 volume of 10% CTAB, it was centrifuged (2,800 rpm, 15 minutes,

room temperature). The surface layer was transferred to a new centrifuge tube and then mixed with an equal volume of cold isopropanol. The thus obtained solvent mixture was then centrifuged (4,500 rpm, 20 minutes, room temperature). After the supernatant had been removed using an aspirator, it was added to 5 ml of a TE buffer solution containing 1 M sodium chloride. This was completely dissolved at 55-60°C. This was mixed thoroughly with 5 ml of frozen isopropanol and the DNA was observed. It was placed on the tip of a chip, transferred to an Eppendorf tube (containing 80% frozen ethanol), and then rinsed. The DNA was then rinsed in 70% ethanol and dried. The dried pellet was dissolved in the appropriate amount of TE buffer solution. Then, 5 ml of RNAase A (10 mg/ml) was added, and reacted for one hour at 37°C; Composition of the 2 x CTAB Solution: 2% CTAB, 0.1 M Tris-HCl (pH8.0), 1.4 M sodium chloride, 1% polyvinylpyrrolidone (PVP); composition of the 10% CTAB solution: 10% CTAB, 0.7 M sodium chloride.

The Southern blot method was performed in the following manner:

(i) DNA Electrophoresis and Alkali Denaturation: After 40 μ g of the chromosomal DNA had been completely digested by the restriction enzyme, the standard method was used, and 1.5% agarose gel electrophoresis was performed (50 V). It was then stained with ethidium bromide and photographed. The gel was then shaken for 20 minutes in 400 ml of 0.25 M HCl, and the liquid removed, and the gel permeated with 400 ml of a denaturing solution (1.5 M NaCl, 0.5 M NaOH by shaking slowly for 45 minutes. Next, the liquid was removed, 400 ml of neutral solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.4) was added, and the solution was shaken slowly for 15 minutes. Then, 400 ml of the neutral solution was again

added, and the solution was shaken slowly again for 15 minutes. (ii) Transfer: After electrophoresis, the DNA was transferred to a nylon membrane (Hybond-N Amersham) using 20 x SSC. The transfer took more than 12 hours. After the
5 blotted membrane was allowed to dry at room temperature for an hour, UV fixing was performed for five minutes. 20 x SSC Composition: 3 M NaCl, 0.3 M sodium citrate. (iii) DNA Probe Preparation: The DNA probe preparation was performed using a Random Prime Labeling Kit (Takara Shuzo Co., Ltd.). Next,
10 the reaction solution was prepared in an Eppendorf tube. After the tube was heated for three minutes to 95°C, it was rapidly cooled in ice. Then, 25 ng of the template DNA and 2 µl of the Random Primer were added to make 5 µl. Then, 2.5 µl 10 x buffer solution, 2.5 µl dNTPs, and 5 µl [α -³²P] dCTP (1.85 MBq, 50 mCi) were added, and H₂O was added
15 to bring the volume of reaction mixture to 24 µl. Then, 1 µl of a Klenow fragment was added and the solution was allowed to stand for 10 minutes at 37°C. It was then passed through a NAP10 column (Pharmacia Co., Ltd.) to prepare the
20 purified DNA. After being heated for three minutes at 95°C, it was rapidly cooled in ice, and used as a hybridization probe. (iv) Hybridization: 0.5% (w/v) SDS was added to the following Pre-hybridization Solution, the membrane in (ii) was immersed in the solution, and pre-hybridization was
25 performed for more than two hours at 42°C. Afterwards, the DNA probe prepared in (iii) was added, and hybridization was performed for more than 12 hours at 42°C. Composition of the Pre-hybridization Solution: 5 x SSC, 50 mM sodium phosphate, 50% (w/v) formamide, 5 x Denhardt's solution
30 (prepared by diluting 100 x Denhardt's solution), 0.1% (w/v) SDS. Composition of the 100 x Denhardt's Solution: 2% (w/v) BSA, 2% (w/v) Ficoll 400, 2% (w/v) polyvinylpyrrolidone (PVP). (v) Autoradiography: After rinsing in the manner described

below, autoradiography was performed using the standard method. It was performed twice for 15 minutes at 65°C in 2 x SSC and 0.1% SDS, and once for 15 minutes at 65°C in 0.1 x SSC and 0.1% SDS.

5

The results of the Southern blot analysis of the genome DNA prepared from the resistant strains are shown in FIG 4. As shown in FIG 4, the presence of the hGT gene was verified in four strains (GT1, 6, 8 and 9).

10

(Example 3). Analysis of the Galactosyltransferase Transformant

The cells of the transformants (GT-1, 6, 8 and 9) and wild-type BY-2 in the fifth through seventh day's culture both were harvested, and then suspended in extraction buffer solution (25 mM Tris-HCl, pH 7.4; 0.25 M sucrose, 1 mM MgCl₂, 50 mM KCl). The cells were ruptured using ultrasound processing (200 W; Kaijo Denki Co., Ltd. Japan) or homogenized. The cell extract solution and the microsome fractions were then prepared according to the method of Schwientek, T. et al. (Schwientek, T. and Ernst, J.F., Gene 145, 299-303, 1994). The expression of the hGT proteins was detected using Western blotting and anti-human galactosyltransferase (GT) monoclonal antibodies (MAB 8628; 1:5000) (Uejima, T. et al., Cancer Res., 52, 6158-6163, 1992; Uemura, M. et al., Cancer Res., 52, 6153-6157, 1992) (provided by Professor Narimatsu Hisashi of Soka University). Next, the blots were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (5% skim milk 1:1000; EY Laboratories, Inc., CA), and a colorimetric reaction using horseradish peroxidase was performed using the POD Immunoblotting Kit (Wako Chemicals, Osaka).

30

An immunoblot analysis of the complex glycans unique to plants was performed using polyclonal antiserum against β -fructosidase in the cell walls of carrots and horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (5% skim milk 1:1000; Sigma) (Lauriere, M. et al., Plant Physiol. 90, 1182-1188, 1989).

The β 1,4-galactosyltransferase activity was assayed as a substrate using UDP-galactose and a pyridylamino (PA-) labeled GlcNAc₂Man₃GlcNAc₂ (GlcNAc₂Man₃GlcNAc₂-PA) (Morita, N. et al., J. Biochem. 103, 332-335, 1988). The enzyme reaction solution contained 1-120 μ g protein, 25 mM sodium cacodylate (pH 7.4), 10 mM MnCl₂, 200 mM UDP-galactose, and 100 nM GlcNAc₂Man₃GlcNAc₂-PA. An HPLC analysis was performed on the reaction product using PALPAK Type R and PALPAK Type N columns (Takara Shuzo Co., Ltd.) and the method recommended by the manufacturer. The GlcNAc₂Man₃GlcNAc₂-PA used as the standard marker was used along with Gal₂GlcNAc₂Man₃GlcNAc₂-PA and two isomers of GalGlcNAc₂Man₃GlcNAc₂-PA purchased from Takara Shuzo Co. Ltd. and Honen Co., Ltd.

The immunoblottings for the proteins derived from the transformant and the wild-type cells are shown in FIG 5. As shown in FIG 5, positive signals of a molecular weight of 50 kDa were observed. This is greater than the molecular weight estimated from the amino acid sequence (40 kDa) and is roughly equivalent to the bovine galactosyltransferase purified from ascites and expressed in yeast (Uemura, M. et al., Cancer Res., 52, 6153-6157, 1992; Schwientek, T. et al., J. Biol. Chem., 271 (7), 3398-3405, 1996). In the microsome fraction, immunoreactive bands (FIG 5, Lanes 1,4) stronger than those of the cell lysate (FIG 5, Lanes 6-8)

were observed. This means that hGT is localized preferentially in the cell. No immunoreactive bands were detected in the wild-type cells.

5 The proteins in the microsome fractions of transformant GT6 and wild-type BY-2 were bound in an RCA₁₂₀ agarose column (Wako Chemicals, Osaka), and then rinsed with 15 volumes of 10 mM ammonium acetate pH 6.0. Next, the bound proteins were
10 eluted using 0.2 M lactose. After separation using SDS-PAGE, the proteins were stained using silver staining (Wako Silver Staining Kit) (FIG 6) or lectin (FIG 7). In the lectin staining, the membrane blots were rinsed in a TTBS buffer solution (10 mM Tris-HCl, pH 7.4; 0.15 M NaCl; 0.05% Tween 20) and incubated with horseradish peroxidase labeled RCA₁₂₀
15 (Honen Co., Ltd.). Galactosylated glycan was then observed using a Immunoblotting Kit (Wako Chemicals, Osaka) (FIG 7). As shown in FIG 7, an RCA₁₂₀ binding was not observed in the wild-type BY2 cells, and the GT6 had a glycoprotein with galactose on the non-reducing terminus of the glycan
20 portion.

The protein extract from the wild-type BY2 cells and the GT6 cells as well as the GT6 proteins eluted from the RCA₁₂₀ affinity chromatography were probed using polyclonal
25 antibodies unique to complex glycan (FIG 8). The antiserum binds predominantly to the β 1,2-xylose residue on the plant glycoprotein (Lauriere, M. et al., Plant Physiol. 90, 1182-1188, 1989). As shown in FIG 8, the wild-type BY2 cells (Lane 1) contain glycoproteins that reacted with the
30 polyclonal antiserum. GT6 contains very few glycoproteins that reacted with the polyclonal antiserum (Lane 2). The GT6 glycoproteins eluted from RCA₁₂₀ affinity chromatography did not bind to the polyclonal antiserum, indicating that

the galactosylated glycan does not contain β 1,2-xylose residue (Lane 3).

(Example 4) Introduction of the Horseradish Peroxidase (HRP) gene to the hGT-Introduced Cultivated Tobacco Cells

Horseradish peroxidase gene was introduced to the resultant GT6 cell line. Among the different types of plant peroxidase, horseradish peroxidase, especially HRP isozyme C, HRP (EC1.11.1.7) has been the subject of extensive research.

HRP can be used in various enzyme reactions because of its superior stability and a broad spectrum of substance specificity. For example, it has been used in enzyme immunology for binding with a secondary antibody in Western blotting. A number of horseradish peroxidase isozyme genes have now been cloned (Fujiyama, K. et al., Eur. J. Biochem., 173, 681-687, 1988 and Fujiyama, K. et al., Gene, 89, 163-169, 1990). ClaPeroxidase (ClaPRX) which is encoded by prxCla is first translated as a protein consisting of 353 amino acids containing an extra peptide consisting of 30 amino acids at the N terminus and 15 amino acids at the C terminus. Then, this is processed to form a mature enzyme with 308 amino acids (Fujiyama, K. et al., Eur. J. Biochem., 173, 681-687, 1988). The molecular weight of ClaPRX ranges between 42,200 and 44,000. Of this molecular weight, sugar chains account for 22-27%, and there are eight N-linked sugar chains (Welinder, K.G., Eur. J. Biochem., 96, 483-502, 1979). The introduction of the ClaPRX gene was performed using the binary vector pBIHm-HRP for HRP expression shown in FIG 9.

The pBIHm-HRP was prepared in the following manner. First, a 1.9 kbp HindIII-SacI fragment was prepared from a vector 35S-prxCla for plant expression, which carries an HRP cDNA (Kawaoka, A. et al., J. Ferment. Bioeng., 78, 49-53, 1994).

The HindIII-SacI fragment contains a full length 1.1 kbp prxCla cDNA following a 0.8 kbp CaMV35S promoter. The 1.9 kbp HindIII-SacI fragment was inserted in the HindIII-SacI site of the binary vector pBI101HmB (Akama, K. et al., Plant Cell Rep., 12, 7-11, 1992). The BamHI site at 3' of the hygromycin resistant gene (HPT gene) had been destroyed.

Because the GT6 strain is kanamycin resistant, the hygromycin-resistant hpt gene was used as the selection marker (Griz, L. and Davies J., Gene, 25, 179-188, 1983). The transformation of the GT6 strain by HRP gene was performed using the method described in Rempel, D.H. and Nelson, L.M. (Rempel, D.H. and Nelson, L.M., Transgenic Res. 4: 199-207, 1995). In order to obtain HRP transformant as a control, an HRP gene was introduced to a wild-type BY2 cell to obtain a BY2-HRP strain. The double-transformant GT6-HRP with hGT and HRP was obtained in which an ordinary transformation process takes place.

(Example 5) Verification of the Expression of HRP in the Cultivated Double-Transformant Tobacco Cells

Double transformant GT6-HRP, control BY2-HRP and wild-type (WT) cell line were examined for the expression of HRP activity using the following method. As seen in Table 1, the HRP gene-introduced transformant had peroxidase activity about five times higher than the wild-type cell line.

Table 1

Clone Number	Specific activity [U/mg protein]
WT-HRP-1	10.3
WT-HRP-5	11.3
WT-HRP-7	12.6

GT-HRP-4	11.1
GT-HRP-5	9.35
GT-HRP-8	9.47
Wild Type	2.49

Clone BY2-HRP obtained by introducing the HRP gene to the wild type expressed the same degree of peroxidase activity as the GT6-HRP double transformant with hGT and HRP.

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(Peroxidase Activity Measurement)

The cultivated tobacco cells were placed into an Eppendorf tube containing Solution D and were ruptured using a homogenizer (Homogenizer S-203, Ikeda Rika Co., Ltd.). The supernatant was collected after centrifugation (12,000 rpm, 20 minutes, 4°C) and then used as the crude enzyme solution. Next, 1 ml of Solution A, 1 ml of Solution B and 2 ml of Solution C were mixed together, and the mixture was incubated for five minutes at 25°C. The crude enzyme solution appropriately diluted with Solution D was added to the mixture, and allowed to react for three minutes at 25°C. The reaction was stopped by the addition of 0.5 ml of 1 N HCl, and the absorbance at 480 nm was measured. As a control, a solution with 1 N HCl added before the introduction of the enzyme was used.

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Solution A: 1 mM o-aminophenol

Solution B: 4 mM H₂O₂

Solution C: 200 mM sodium phosphate buffer (pH 7.0)

25 Solution D: 10 mM sodium phosphate buffer (pH 6.0)

Next, in order to determine whether or not the rise in peroxidase activity was due to the expression of HRP, activity staining was performed after separation by gel isoelectric focusing. The isoelectric focusing was

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performed using a BIO-RAD Model 111 Mini-IEF Cell. The hydrophobic surface of the PAGE gel support film was attached to a glass plate, and then placed on a casting tray. The prepared gel solution was poured between the support film and the casting tray and then photopolymerized for 45 minutes under a fluorescent lamp. The sample was applied to the gel, and the gel was positioned so as to come into contact with both graphite electrodes wetted with distilled water in the electrophoretic bath. Electrophoresis was then performed for 15 minutes at 100 V, 15 minutes at 200 V and 60 minutes at 450 V. Composition of the Gel Solution (per 1 Gel Sheet): distilled water 2.75 ml, acrylamide (25%T, 3%C) 1.0 ml, 25% glycerol 1.0 ml, Bio-lite (40%, pH 3-10) 0.25 ml, 10% ammonium persulfate 7.5 μ l, 0.1% sodium riboflavin5'-phosphate 25 μ l, TEMED 1.5 μ l.

The activity staining of peroxidase was performed according to the method of Sekine et al. (Sekine et al., Plant Cell Technology, 6, 71-75, 1994). As shown in FIG 10, a significant band not found in wild-type cell line was detected in the pI 7.8 position in the BY2-HRP cell line and the GT6-HRP strain. The results of a Western analysis using anti-HRP antibodies confirmed the detection of a signal at the position corresponding to pI 7.8 and the expression of HRP in the double transformant GT6-HRP with hGT and HRP.

(Example 6) Structural Analysis of the N-linked Sugar Chains in the Transformant GT6 Cells

(Method Used to Analyze the Sugar Chain Structure)

The N-linked sugar chains in the transformant GT6 cells were analyzed by combining reverse-phase HPLC and size-fractionation HPLC, performing the two-dimensional PA sugar

chain mapping, performing exoglycosidase digestion, and then performing ion spray tandem mass spectrometry (IS-MS/MS) (Perkin Elmer Co., Ltd.). First, the cell extract solution was delipidated with acetone, treated with
5 hydrazine for 12 hours at 100°C, and the sugar chain portion was released. The hydrazinolysate was N-acetylated, desalted using the Dowex 50X2 and the Dowex 1X2 (The Dow Chemical Co., Ltd. and its representative in Japan, Muromachi Chemical Industry Co., Ltd.), then fractionized
10 by using 0.1 N ammonia and the Sephadex G-25 gel filtration column (1.8 x 180 cm) (Pharmacia Co., Ltd.). Pyridylation was then performed as described above. The pyridylaminated sugar chains (PA sugar chains) were then separated using a Jasco 880-PU HPLC device with a Jasco
15 821-FP Intelligent Spectrophotometer (Japan Spectroscopic Co., Ltd.) and Cosmosil 5C18-P and Asahipak NH2P-50 columns. The elution positions were compared with a standard either produced by the applicant or purchased (from Wako Pure Chemical Industries, Ltd. and Takara Shuzo Co., Ltd.).

20 The glycosidase digestion using N-acetyl- β -D-glucosaminidase (*Diplococcus pneumoniae*, Boehringer Mannheim) or mannosidases (Jack bean, Sigma) was performed on about 1 nmol of the PA sugar chains under the same
25 conditions as the method described in Kimura, Y. et al., Biosci. Biotech. Biochem. 56 (2), 215-222, 1992. Digestion using β -galactosidase (*Diplococcus pneumoniae*, Boehringer Mannheim) or *Aspergillus saitoi*-derived α -1,2 mannosidase (provided by Dr. Takashi Yoshida at Tohoku University) was
30 performed by adding 1 nmol of PA sugar chains and 200 mU β -galactosidase or 60 μ g of α -1,2 mannosidase to 50 mM of sodium acetate buffer (pH 5.5) and incubating at 37°C. After the resultant reaction solution was boiled and the enzyme

reaction was stopped, a portion of the digested product was analyzed using size-fractionation HPLC. The molecular weight of the digested product was analyzed using ion spray tandem mass spectrometry (IS-MS/MS) and/or compared to the standard sugar chain as described in Palacpac, N.Q. et al., Biosci. Biotech. Biochem. 63(1) 35-39, 1999 and Kimura, Y. et al., Biosci. Biotech. Biochem. 56 (2), 215-222, 1992.

The IS-MS/MS experiment was performed using a Perkin Elmer Sciex API-III. It was performed in positive mode with an ion spray voltage of 4200 V. Scanning was performed every 0.5 Da, and the m/z was recorded from 200.

(Analysis of the Sugar Chains in the GT6 Cells)

The PA sugar chains prepared from the GT6 cells were purified and analyzed using a combination of reverse-phase HPLC and size-fractionation HPLC. In Fraction I at the 10-20 minute positions in the size-fractionation HPLC (FIG 11), no N-linked sugar chains were eluted. This suggests that the Fraction I is a non-absorption portion containing by-products of hydrazinolysis. In the MS/MS analysis, no fragment ion with m/z values of 300, which corresponds to PA-GlcNAc, was detected. Similarly, Fraction XI at the 50-60 minute positions did not have a peak indicating elution by the size-fractionation HPLC. Therefore, it is clear that there were no N-linked sugar chains. The 17 peaks including A-Q shown in FIG 12 were all collected and purified after the analysis from Fraction II to Fraction X in the size-fractionation HPLC (FIG 11) was completed.

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The IS-MS/MS analysis found that seven of these peaks were N-linked sugar chains. The following is the result from the analysis of these peaks.

The elution positions and molecular weights of oligosaccharides -A, -E, -H, -I, -M, -O, -P and -Q (FIG 12) did not correspond to those of PA sugar chain standards. In the MS/MS analysis, the m/z values of 300 and 503, which respectively correspond to PA-GlcNAc and PA-GlcNAc₂, were detected. However, the fragment ions were not detected corresponding to ManGlcNA₂ (M1) or the trimannose core sugar chain Man₃GlcNAc₂ (M3) which are generally found in N-linked sugar chain (data not shown). Even the oligosaccharides -B, -D and -N at the other peaks did not have fragment ions detected with an m/z value of 300. Thus, these were not N-linked sugar chains. The seven remaining N-linked sugar chains were then examined.

The elution positions and molecular weights of peak-C (m/z 1637.5; molar ratio 9.3%), peak-F ([M+2H]²⁺ + m/z 819.5, [M+H]⁺ + m/z 1639; molar ratio 15.9%), and peak-G (m/z 1475.5; molar ratio 19.5%) indicated high mannose-type sugar chains Man₇GlcNAc₂ (Isomer M7A and M7B) and Man₆GlcNAc₂ (M6B) respectively. When digested by Jack bean α -mannosidase, it was indicated that the N-linked sugar chains are degraded to ManGlcNAc (M1) by size-fractionation HPLC analysis (data not shown). In an IS-MS experiment on the digestion product, the ion with an m/z value of 665.5 corresponding to a calculated value of 664.66 for M1 was detected, indicating that these N-linked sugar chains have the same structure as respective corresponding PA sugar chain standard.

Peak-J (6.6%) had a molecular weight of 1121.5, which is almost the same as the calculated molecular weight value of m/z 1121.05 of Man₃Xyl₁GlcNAc₂-PA (M3X). The positions of the fragment ions were 989.5, 827.5, 665.5, 503.3 and

300. This does not contradict the findings that Xyl, Man, Man, Man, and GlcNAc were released in successive order from Man₃Xyl₁GlcNAc₂-PA. When digested using Jack bean α -mannosidase, the mannose residues on the non-reducing terminus can be removed, and the two-dimensional mapping revealed the same elution positions as those of Man₁Xyl₁GlcNAc₂-PA (data not shown).

The results of the analysis of the IS-MS experiment on peak-K (13.2%) fraction revealed that this fraction contains two types of N-linked sugar chains, one has the molecular weight of 1314.0 (1.4%) and the other has the molecular weight of 1354.5 (11.8%). This fraction was subjected to reverse-phase HPLC, purified and analyzed. The sugar chain peak K-1 with a molecular weight of 1314.0 had the same two-dimensional mapping and m/z value measured as that of the sugar chain standard Man₃GlcNAc₂-PA (M5). When treated using jack bean α -mannosidase, the elution positions of the degraded product had shifted to positions similar to those of M1 in the two-dimensional mapping. This indicates the removal of four mannose residues.

(Galactose-added N-linked Type Sugar Chains in the GT6 Cells)

The determined m/z value of 1354.5 for sugar chain peak K-2 is almost the same as the molecular weight m/z value of 1354.3 predicted for Gal₁GlcNAc₁Man₃GlcNAc₂-PA (GalGNM3). The result of the mass spectrometry indicated that fragment ions were derived from the parent molecules. The m/z value of 1193.5 indicated GlcNAc₁Man₃GlcNAc₂-PA, the m/z value of 989.5 indicated Man₃GlcNAc₂-PA, the m/z value of 827.5 indicated Man₂GlcNAc₂-PA, the m/z value of 665 indicated ManGlcNAc₂-PA, the m/z value of 503 indicated GlcNAc₂-PA,

the m/z value of 336 indicated ManGlcNAc, the m/z value of 300 indicated GlcNAc-PA, and the m/z value of 204 indicated GlcNAc. From the putative N-linked sugar chain structure, it is considered to be either of two GalGNM3 isomers (FIG 13). It is either Gal β 4GlcNAc β 2Man α 6(Man α 3)Man β 4GlcNAc β 4GlcNAc-PA or Man α 6(Gal β 4GlcNAc β 2Man α 3)Man β 4GlcNAc β 4GlcNAc-PA. The purified PA sugar chains had reverse-phase HPLC elution positions that were the same as the sugar chain standard Man α 6(Gal β 4GlcNAc β 2Man α 3) Man β 4GlcNAc β 4GlcNAc-PA (FIG 13B).

The sugar chain was treated with exoglycosidase and the structure of the sugar chain was verified. The *D. pneumoniae* β -galactosidase is a Gal β 1,4GlcNAc linkage specific enzyme. The digested product of the sugar chain by the enzyme was eluted at the same position as that of the GlcNAc₁Man₃GlcNAc₂-PA in the size-fractionation HPLC (FIG 14A-II). An m/z of 1192.0 was obtained from the IS-MS/MS analysis. These results indicate a galactose residue has been removed from the GlcNAc on the non-reducing terminus with the β 1,4 binding. When the product was digested by a N-acetyl- β -D-glucosaminidase derived from *Diplococcus pneumoniae*, which is β 1,2 GlcNAc linkage specific (Yamashita, K. et al., J. Biochem. 93, 135-147, 1983), the digested product was eluted at the same position as that of the standard Man₃GlcNAc₂-PA in the size-fractionation HPLC (FIG 14A-III). When the digested product was treated with jack bean α -mannosidase, it was eluted at the same position as that of the standard ManGlcNAc₂-PA in the size-fractionation HPLC (FIG 14A-IV). The sugar chain structure is shown in K-2 of FIG 15.

The mass spectroscopy analysis of Peak L(35.5%) gave [M+2H]

2+ of 840, [M+H]⁺ of 1680.0, which nearly matched the molecular weight m/z value of 1678.55 expected for Gal₁GlcNAc₁Man₅GlcNAc₂-PA (GalGNM5). The result of the mass spectrometry indicated fragment ions derived from the parent molecules. The m/z value of 1313.5 indicated Man₅GlcNAc₂-PA, the m/z value of 1152 indicated Man₄GlcNAc₂-PA, the m/z value of 989.5 indicated Man₃GlcNAc₂-PA, the m/z value of 827.5 indicated Man₂GlcNAc₂-PA, the m/z value of 665 indicated ManGlcNAc₂-PA, the m/z value of 503 indicated GlcNAc₂-PA, the m/z value of 336 indicated ManGlcNAc, the m/z value of 300 indicated GlcNAc-PA, and the m/z value of 204 indicated GlcNAc. The product digested with *D. pneumoniae* β-galactosidase was eluted at the same position as that of GlcNAc₁Man₅GlcNAc₂-PA in the size-fractionation HPLC (FIG 14B-II). The results indicate that a galactose residue is bound to the GlcNAc on the non-reducing terminus with the β1,4 linkage. The removal of the galactose was confirmed by the molecular weights obtained from the IS-MS/MS analysis. [M+2H]²⁺ was 759 and [M+H]⁺ was 1518.0. The mass spectrometry indicated fragments ions derived from the GlcNAc₁Man₅GlcNAc₂-PA with a parent signal of m/z 1518.0. The m/z value of 1314 indicated Man₅GlcNAc₂-PA, the m/z value of 1152 indicated Man₄GlcNAc₂-PA, the m/z value of 990 indicated Man₃GlcNAc₂-PA, the m/z value of 827.5 indicated Man₂GlcNAc₂-PA, the m/z value of 665.5 indicated Man₁GlcNAc₂-PA, the m/z value of 503 indicated GlcNAc₂-PA, and the m/z value of 300 indicated GlcNAc-PA. When the GlcNAc₁Man₅GlcNAc₂-PA was digested with an N-acetyl-β-D-glucosaminidase derived from *Diplococcus pneumoniae*, the digested product was eluted at the same position as that of the standard Man₅GlcNAc₂-PA in the size-fractionation HPLC (FIG 14B-III). Even when treated with α-1,2 mannosidase derived from *Aspergillus saitoi*, the elution

position did not shift (FIG 14B-IV). However, when treated with jack bean α -mannosidase, it was eluted at the same position as that of standard $\text{Man}_1\text{GlcNAc}_2\text{-PA}$ in the size-fractionation HPLC (FIG 14B-V). This indicates the removal of four mannose residues in the non-reducing terminus. These results indicate that in the PA sugar chain, none of five mannose residues are $\alpha 1,2$ linked to the mannose residue which are $\alpha 1,3$ binding. The exoglycosidase digestion, two-dimensional sugar chain mapping, and IS-MS/MS analysis indicate a sugar chain structure of GalGNM5 as shown by L in FIG 15.

FIG 20 summarizes the above results regarding the structure of N-linked glycans and the ratio of each N-linked glycan in GT6 cell line along with those in wild-type BY2 cell line determined similarly. In FIG 20, \square denotes GlcNAc, \circ denotes mannose, \bullet denotes galactose, \square with hatched lines therein denotes xylose, and \circ with dots therein denotes fucose respectively.

In GT6 cell line, the isomers Man7- , Man6- and Man5GlcNAc_2 were observed. Because those high-mannose type oligosaccharides will be substrates for β 1,4-galactosyltransferase (Gal T), introduction of GlcNAc I, Man I and Man II cDNAs can more efficiently lead the oligosaccharide Man7-5GlcNAc_2 to $\text{GlcNAcMan3GlcNAc}_2$, which can be a substrate of GalT (FIG 21).

A. thaliana *cg1I* mutant, that lacks GnT I, can not synthesize complex type N-glycans (von Schaewen, A., Sturm, A., O'Neill, J., and Chrispeels, MJ., Plant Physiol., 1993 Aug;102(4):1109-1118, Isolation of a mutant Arabidopsis plant that lacks N-acetyl glucosaminyl transferase I and

is unable to synthesize Golgi-modified complex N-linked glycans). Complementation with the human GnT I in the *cglI* mutant indicated that the mammalian enzyme could contribute the plant N-glycosylation pathway (Gomez, L. and Chrispeels, M.J., Proc. Natl. Acad. Sci. USA 1994 March 1;91(5):1829-1833, Complementation of an *Arabidopsis thaliana* mutant that lacks complex asparagine-linked glycans with the human cDNA encoding N-acetylglucosaminyltransferase I.) Furthermore, GnT I cDNA isolated from *A. thaliana* complemented N-acetylglucosaminyltransferase I deficiency of CHO Lec1 cells (Bakker, H., Lommen, A., Jordi, W., Stiekema, W., and Bosch, D., Biochem. Biophys. Res. Commun., 1999 Aug 11;261(3):829-32, An *Arabidopsis thaliana* cDNA complements the N-acetylglucosaminyltransferase I deficiency of CHO Lec1 cells). cDNAs encoding human Man I and Man II were isolated and sequenced (Bause, E., Bieberich, E., Rolfs, A., Volker, C. and Schmidt, B., Eur J Biochem 1993 Oct 15;217(2):535-40, Molecular cloning and primary structure of Man9-mannosidase from human kidney; Tremblay, L.O., Campbell, Dyke, N. and Herscovics, A., Glycobiology 1998 Jun;8(6):585-95, Molecular cloning, chromosomal mapping and tissue-specific expression of a novel human alpha 1,2-mannosidase gene involved in N-glycan maturation; and Misago, M., Liao, Y.F., Kudo, S., Eto, S., Mattei, M.G., Moremen, K.W., Fukuda, M.N., Molecular cloning and expression of cDNAs encoding human alpha-mannosidase II and a previously unrecognized alpha-mannosidase IIx isozyme). Human Man I has two isozymes, Man IA and Man IB, and the nucleotide structure of isozymes' cDNA was shown (Bause, E., et al., and Tremblay, L.O., *supra*). By transforming these cDNAs into the BY cell line, an efficient cell line producing human-type glycoprotein, can

be obtained. β 1,4-Galactosyltransferase (Gal T) uses UDP-galactose as a donor substrate and GlcNAc2Man3GlcNAc2 as an acceptor substrate. Efficient supply of UDP-galactose will enhance the Gal T enzyme reaction, and more galactosylated oligosaccharide will be produced (FIG 22).

(Example 7) Structural Analysis of the Sugar Chains on the HRP in the Double Transformant GT6-HRP Cells

A crude cell lysate was obtained from the homogenate of 50 g of cultured GT6-HRP cells or control BY2-HRP cells grown for seven days, respectively. This crude cell lysate solution was applied to a CM Sepharose FF column (1 x 10 cm) (Pharmacia Co., Ltd.) equilibrated with 10 mM of sodium phosphate buffer (pH 6.0). After washing the column, the eluted peroxidase was measured at an absorbance of 403 nm. The pooled fraction was concentrated using an ultrafilter (molecular weight cut off: 10,000, Advantec Co., Ltd.), dialyzed against 50 mM of a sodium phosphate buffer (pH 7.0), and then applied to an equilibrated benzhydroxaminic acid-agarose affinity column (1 x 10 cm) (KemEn Tech, Denmark). After the column was washed in 15 volumes of 50 mM of sodium phosphate buffer (pH 7.0), the absorbed HRP was eluted using 0.5 M boric acid prepared in the same buffer. The peroxidase active fraction obtained was then pooled, dialyzed, and concentrated.

The purified HRP prepared from the double transformant GT6-HRP cells or BY2-HRP cells was applied to a 1 x 10 cm RCA₁₂₀-agarose column. The column was then washed with 15 volumes of 10 mM ammonium acetate (pH 6.0). The absorbed proteins were then eluted and assayed using conventional methods.

Lectin staining was then performed on the purified HRP eluted from RCA₁₂₀ affinity chromatography whose specificity is specific to β 1,4 linkage galactose. The lectin RCA₁₂₀ was bound to only the HRP produced by the transformed cell GT6-HRP. Because the lectin binding was dramatically reduced by preincubation with the galactose which can compete with the lectin (FIG 16b-III), the binding is carbohydrate specific. Even when the purified HRP is pre-treated with *D. pneumoniae* β -galactosidase, the RCA₁₂₀ binding was inhibited. These results indicate RCA bound specifically to β 1,4-linked galactose at the non-reducing end of N-linked glycan on HRP. The absence of RCA-bound glycoproteins in the BY2-HRP cells indicates that these cells can not transfer the β 1,4 linked galactose residue to the non-reducing terminus of the HRP glycan.

Reverse-phase HPLC of PA derivatives derived from HRP purified using RCA₁₂₀ indicated that the sugar chains on the HRP proteins purified from the GT6-HRP appear as a single peak (FIG 17). In the reverse phase HPLC, a Cosmosil 5C18-P column or Asahipak NH2P column was used in a Jasco 880-PU HPLC device with a Jasco 821-FP Intelligent Spectrofluorometer. Neither bound proteins nor detectable peaks were observed in the HRP fractions purified from BY2-HRP. The peak obtained from the GT6-HRP in the size-fractionation chromatography was homogenous. The two-dimensional mapping analysis of the peak and chromatography of the peak at the same time with standard sugar chain indicated that the oligosaccharide contained in the peak was Gal₁GlcNAc₁Man₅GlcNAc₂-PA. The confirmation of this structure was provided using continuous exoglycosidase digestion. The standard sugar chains used were a sugar chains prepared previously (Kimura, Y. et al.,

Biosci. Biotech. Biochem. 56 (2), 215-222, 1992) or purchased (Wako Pure Chemical, Industries, Ltd. Osaka and Takara Shuzo Co., Ltd.).

- 5 The PA sugar chain digested with β -galactosidase (*D. pneumoniae*) matched the elution position of the standard GlcNAc₁Man₅GlcNAc₂-PA indicating the removal of a galactose residue β 1,4-linked to a non-reducing terminal GlcNAc. Further digestion with *D. pneumoniae* N-acetyl- β -D-glucosaminidase of β -galactosidase-digested products
- 10 produced a sugar chain equivalent which is eluted at the same elution position of Man₅GlcNAc₂-PA, indicating the removal of a GlcNAc residue β 1,2 linked to a non-reducing terminal mannose residue. The removed GlcNAc residue is
- 15 believed to be linked to α 1,3 mannose linked to a β 1,4 mannose residue in view of the N-linked type processing route of the plant. In order to confirm the linkage position of the GlcNAc residue, Man₅GlcNAc₂-PA (M5) was incubated with α 1,2 mannosidase derived from *Aspergillus saitoi*. As
- 20 expected, an elution position shift was not detected, confirming M5 has the structure Man α 1-6(Man α 1,3)Man α 1-6 (Man α 1,3)Man β 1,4GlcNAc β 1,4GlcNAc as predicted. When the sugar chain was digested using jack bean α -mannosidase, it was eluted at the same elution positions
- 25 of known Man₁GlcNAc₂-PA. Therefore, the sugar chain structure corresponded to Man α 1-6(Man α 1,3)Man α 1-6(Gal β 1,4GlcNAc β 1,2Man α 1,3)Man β 1,4GlcNAc β 1,4GlcNAc (Gal₁GlcNAc₁Man₅GlcNA₂). These results indicate that the sugar chain in GT6 cell has the structure shown in FIG 15
- 30 and that the sugar chain structure on an HRP protein derived from the double transformant GT6-HRP is Man α 1-6(Man α 1,3)Man α 1-6(Gal β 1,4GlcNAc β 1,2Man α 1,3)Man β 1,4GlcNAc β 1,4GlcNAc (Gal₁GlcNAc₁Man₅GlcNA₂).

Similarly, the galactosylated N-glycan on HRP derived from the transformant GT6-HRP cells did not react with an antiserum which has been shown to specifically react with β 1,2 xylose residue indicative of plant N-glycans. This indicates that one of the sugar residues shown to be antigenic in complex plant glycan, i.e., xylose residue, is not present (Garcia-Casado, G. et al., Glycobiology 6 (4): 471,477, 1996) (FIG 18).

INDUSTRIAL APPLICABILITY

The present invention provides a method for manufacturing a glycoprotein with a human-type sugar chain. It also provides plant cells that have a sugar chain adding mechanism able to perform a reaction in which a galactose residue is transferred to a acetylglucosamine residue on the non-reducing terminal, wherein the sugar chain adding mechanism is capable of joining a sugar chain which contains a core sugar chain and an outer sugar chain, wherein the core sugar chain consists essentially of a plurality of mannose and acetylglucosamine, and the outer sugar chain contains a terminal sugar chain portion containing a galactose on the non-reducing terminal. The present invention further provides a glycoprotein with a human-type sugar chain obtained by the present invention. A glycoprotein with a mammalian, e.g., human-type sugar chain of the present invention is not antigenic because the glycosylation is a human-type. Therefore, it can be useful for administering to animals including humans.

CLAIMS

1. A method of manufacturing a glycoprotein having a human-type sugar chain, comprising a step in which a transformed plant cell is obtained by introducing to a plant cell the gene of glycosyltransferase and the gene of an exogenous glycoprotein, and a step in which the obtained transformed plant cell is cultivated.
2. A method according to claim 1, wherein the glycosyltransferase is an enzyme capable of conducting a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue.
3. A method according to Claim 1, wherein the glycoprotein with a human-type sugar chain comprises a core sugar chain and an outer sugar chain, wherein the core sugar chain comprises a plurality of mannose and acetylglucosamine, and wherein the outer sugar chain contains a terminal sugar chain portion with a non-reducing terminal galactose.
4. A method according to Claim 3, wherein the outer sugar chain has a straight chain configuration.
5. A method according to Claim 3, wherein the outer sugar chain has a branched configuration.
6. A method according to Claim 5, wherein the branched sugar chain portion has a mono-, bi-, tri- or tetra configuration.
7. A method according to Claim 1 through Claim 6, wherein the glycoprotein contains neither fucose nor xylose.

8. A plant cell having a sugar chain adding mechanism which can conduct a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue, wherein the sugar chain adding mechanism adds a sugar chain containing a core sugar chain and an outer sugar chain, wherein the core sugar chain comprises a plurality of mannose and acetylglucosamine, and wherein the outer sugar chain contains a terminal sugar chain portion with a non-reducing terminal galactose.
9. A plant cell according to claim 8, wherein the plant cell is transformed with the gene of a first enzyme capable of conducting a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue and the gene of a second enzyme which can enhance the first enzyme.
10. A plant cell according to claim 9, wherein the second enzyme is selected from the group consisting of Mannosidase I, Mannosidase II, β 1,4-Galactosyltransferase (GalT) and N-acetylglucosaminyltransferase I (GlcNAcI).
11. A plant regenerated from the plant cell of claim 8.
12. A recombinant plant, or portion thereof, that produces mammalian-like glycoproteins comprising neither fucose or xylose.
13. A glycoprotein with a human-type sugar chain obtained using the method according to Claim 1 through Claim 7.
14. A plant-produced glycoprotein comprising neither fucose or xylose.

PCT

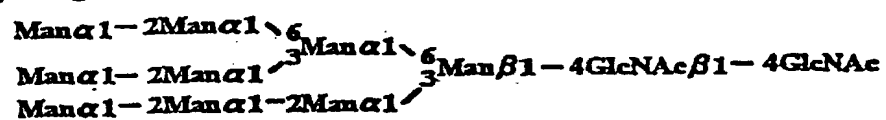
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International Bureau



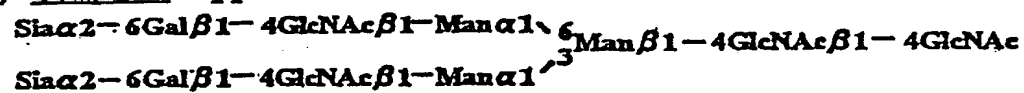
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(21) International Application Number: PCT/JP99/06881 (22) International Filing Date: 8 December 1999 (08.12.99) (30) Priority Data: 10/350584 9 December 1998 (09.12.98) JP (71)(72) Applicants and Inventors: SEKI, Tatsuji [JP/JP]; 5-13-53-209, Mino, Mino-shi, Osaka 562-0001 (JP). FU- JIYAMA, Kazuhito [JP/JP]; A18-308, 1-28, Yamadanishi, Suita-shi, Osaka 565-0824 (JP). (72) Inventor; and (75) Inventor/Applicant (for US only): YOSHIDA, Toshiomi [JP/JP]; A1-505, 2-4, Yamadanishi, Suita-shi, Osaka 565-0824 (JP). (74) Agent: YAMAMOTO, Shusaku; Crystal Tower, 15th Floor, 2-27, Shiromi 1-chome, Chuo-ku, Osaka-shi, Osaka 540-6015 (JP).			(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: A METHOD FOR MANUFACTURING GLYCOPROTEINS HAVING HUMAN-TYPE GLYCOSYLATION (57) Abstract The present invention provides a method for manufacturing a glycoprotein having a human-type sugar chain comprising a step in which a transformed plant cell is obtained by introducing to a plant cell the gene of glycosyltransferase and the gene of an exogenous glycoprotein, and a step in which the obtained transformed plant cell is cultivated.			

1) High Mannose-Type



2) Complex-Type



3) Hybrid-Type

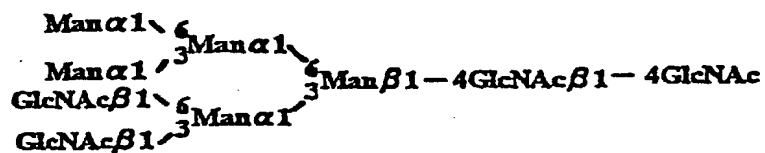
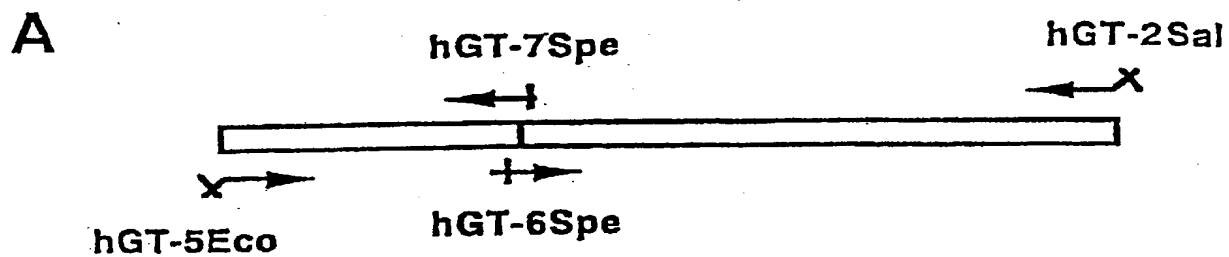


FIG 1



B

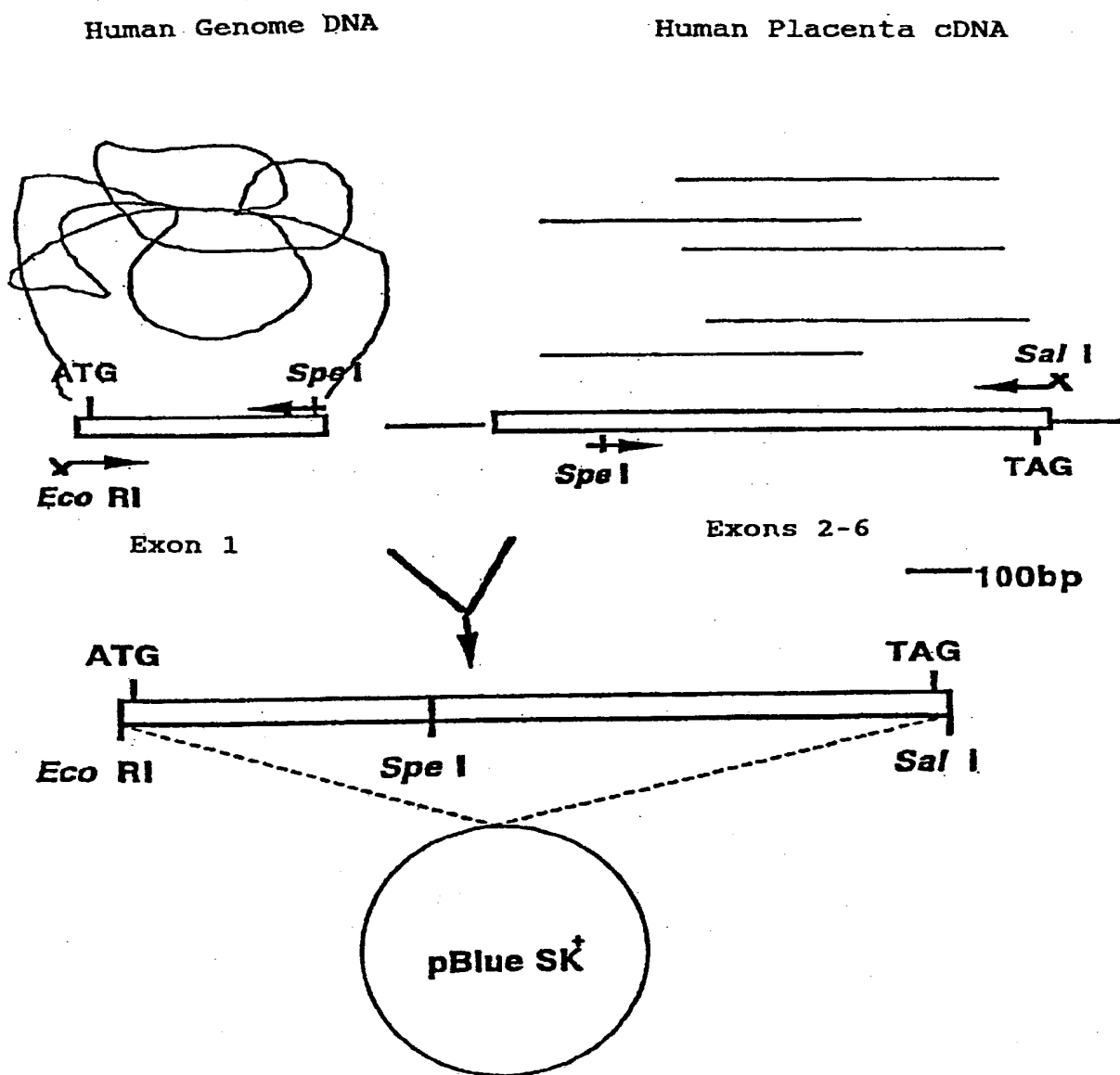


FIG 2

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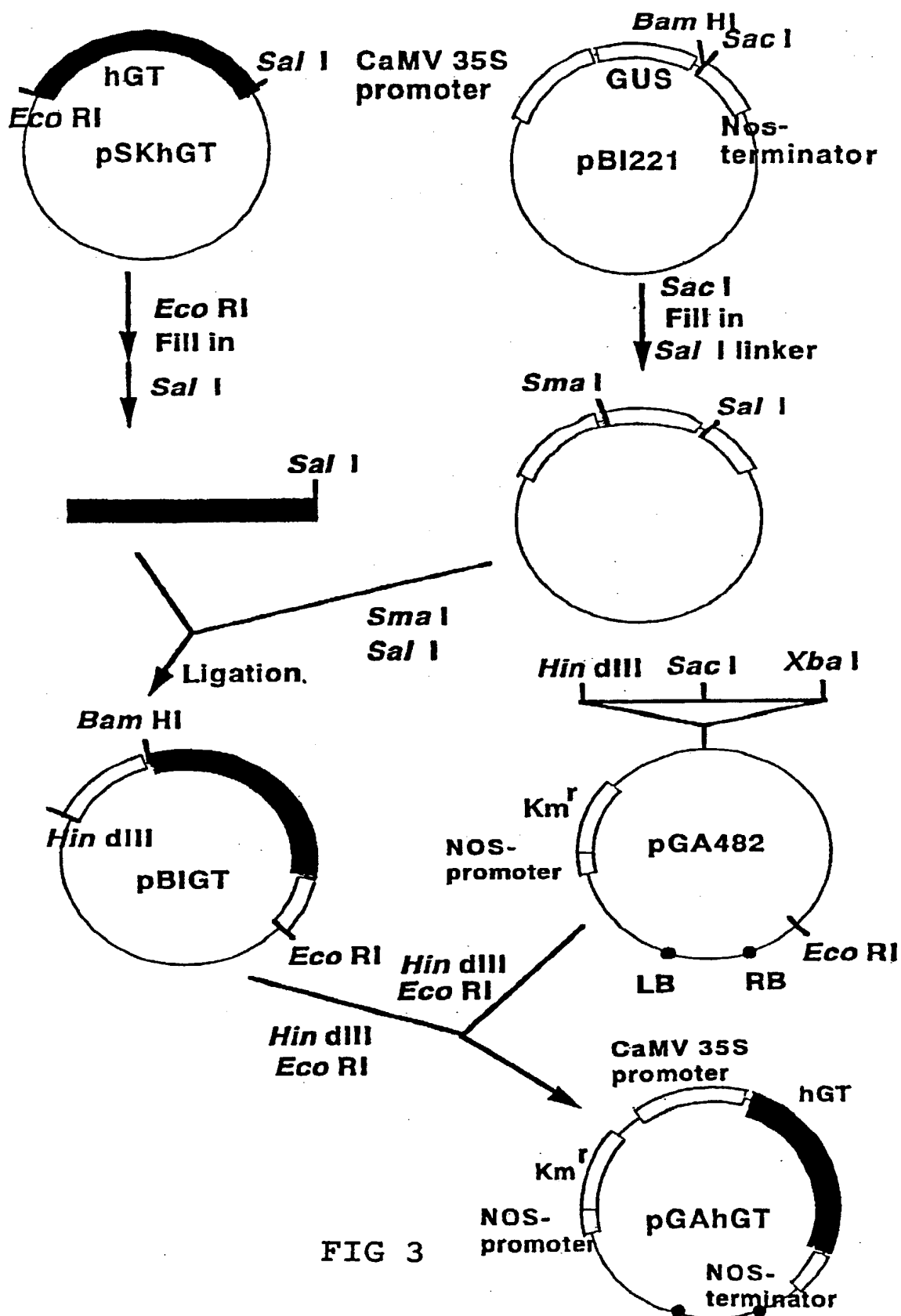


FIG 3

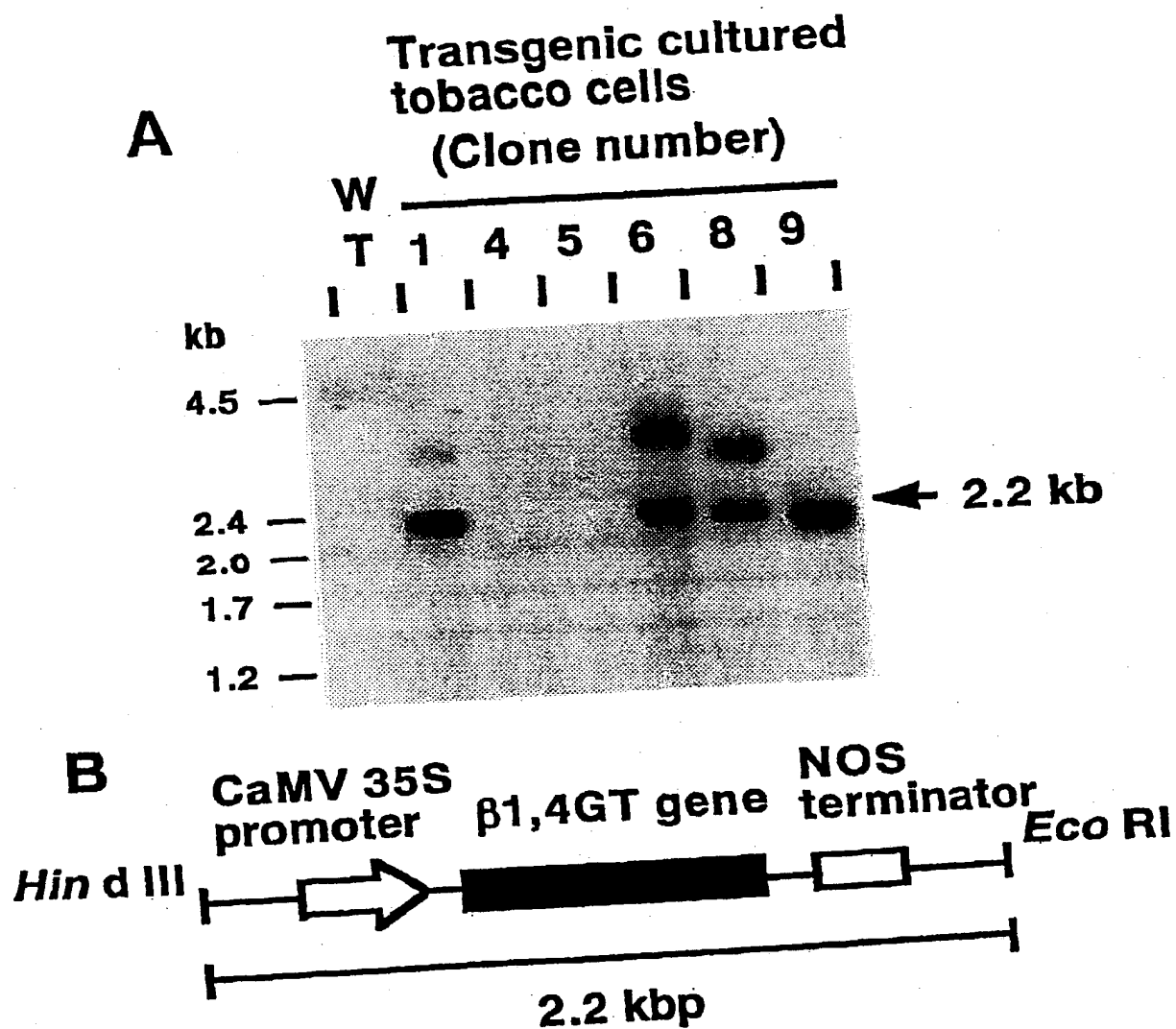


FIG 4

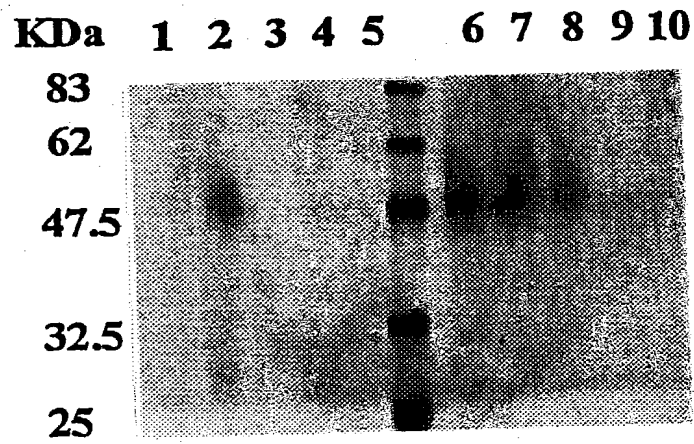


FIG 5

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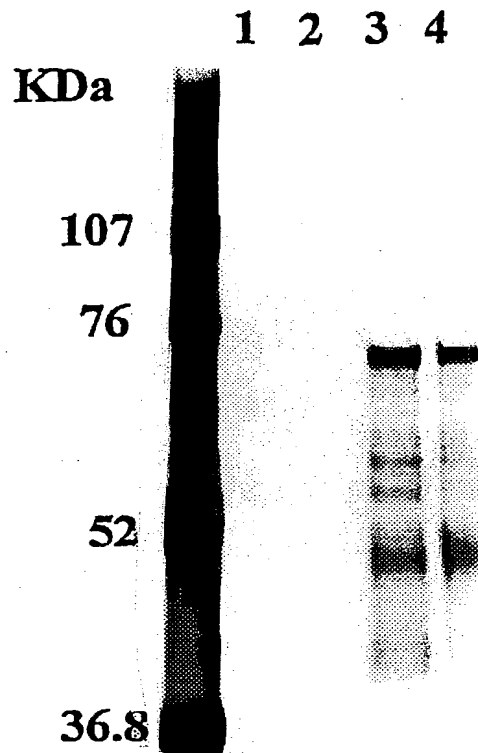


FIG 6

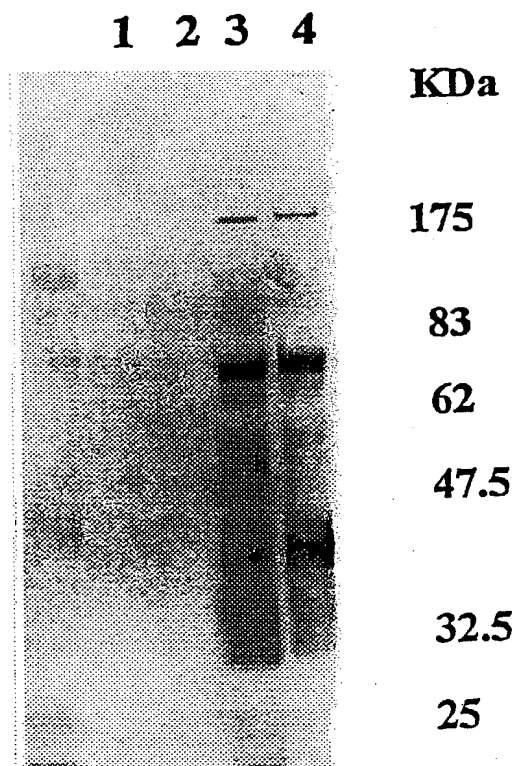


FIG 7

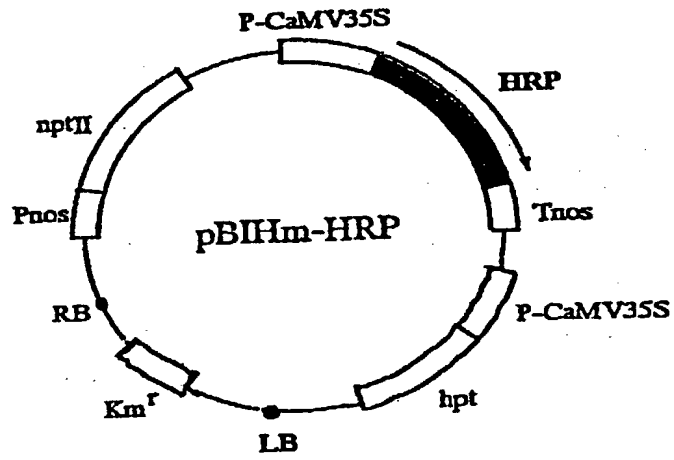


FIG 9

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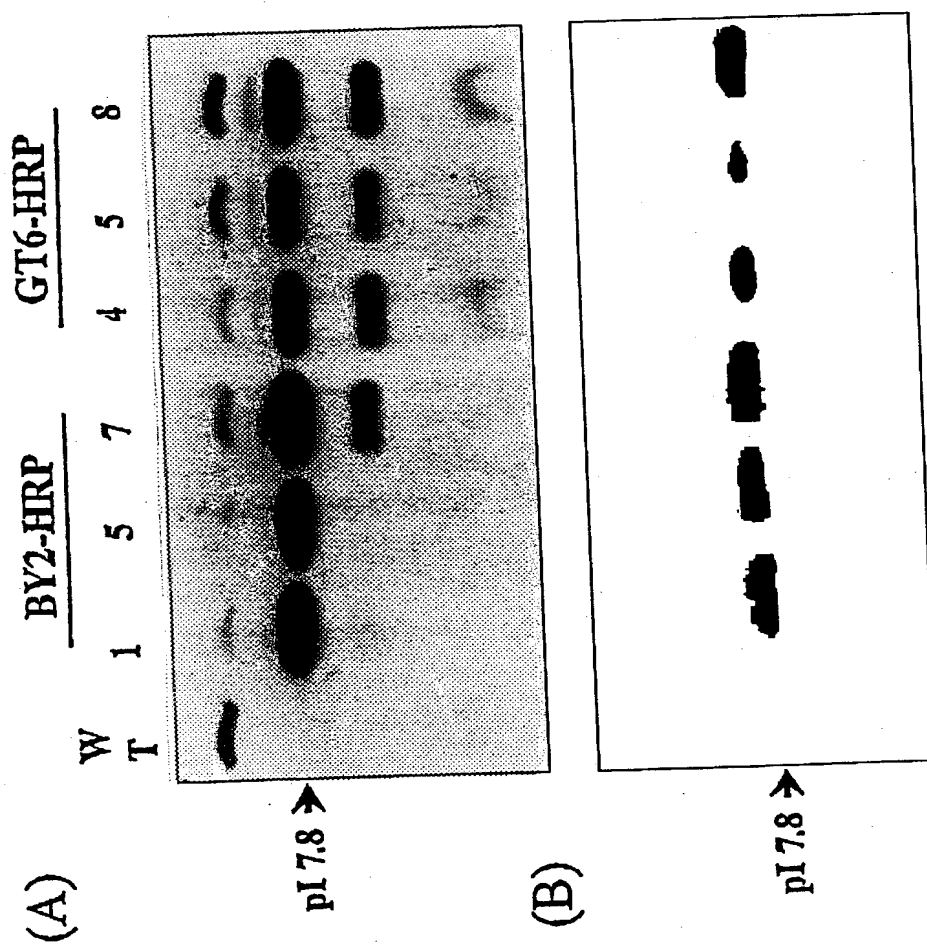


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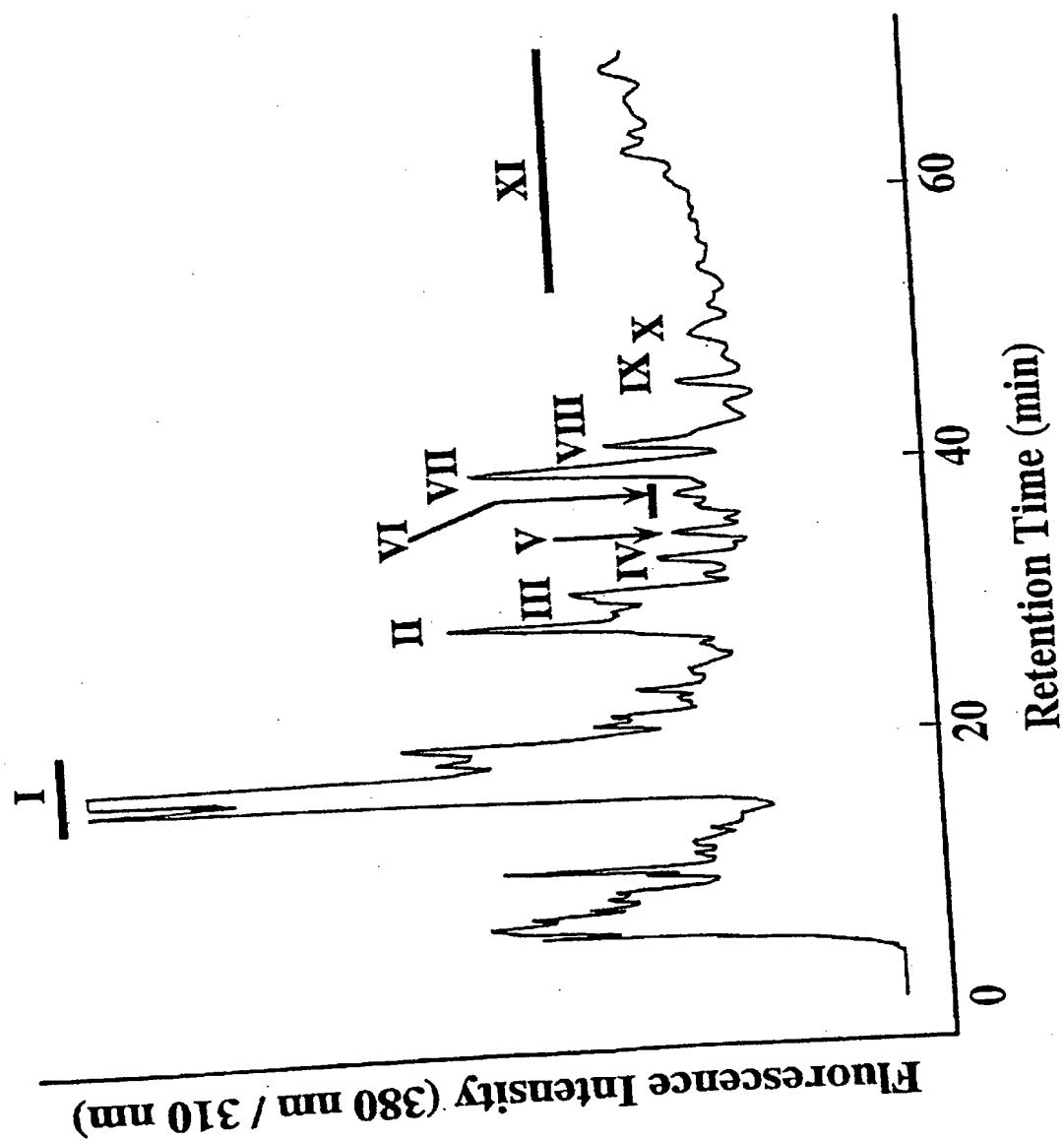


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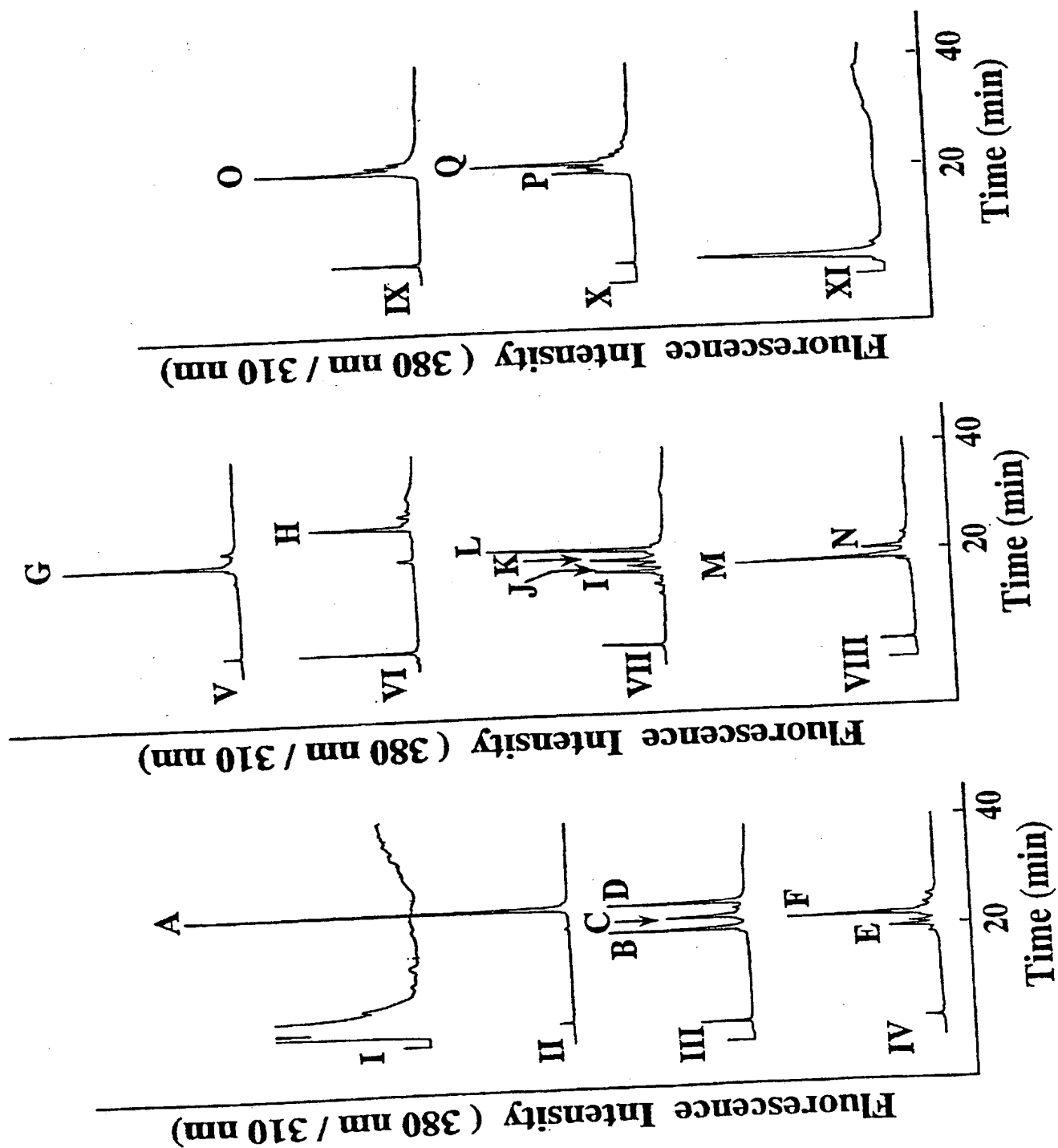


FIG 12

The figure displays an HPLC chromatogram and the chemical structures of two oligosaccharides, A and B.

Chromatogram: The x-axis represents Retention Time (min) from 0 to 60. The y-axis represents Fluorescence Intensity (380 nm/310 nm). A major peak is observed at approximately 45 minutes, with two arrows labeled 'A' and 'B' pointing to it. A smaller peak is visible at approximately 5 minutes.

Chemical Structures:

A: Gal β 4GlcNAc β 2-Man α 6
 Man β 4GlcNAc β 4GlcNAc-PA
 Man α 3

B: Gal β 4GlcNAc β 2-Man α 6
 Man β 4GlcNAc β 4GlcNAc-PA
 Man α 3

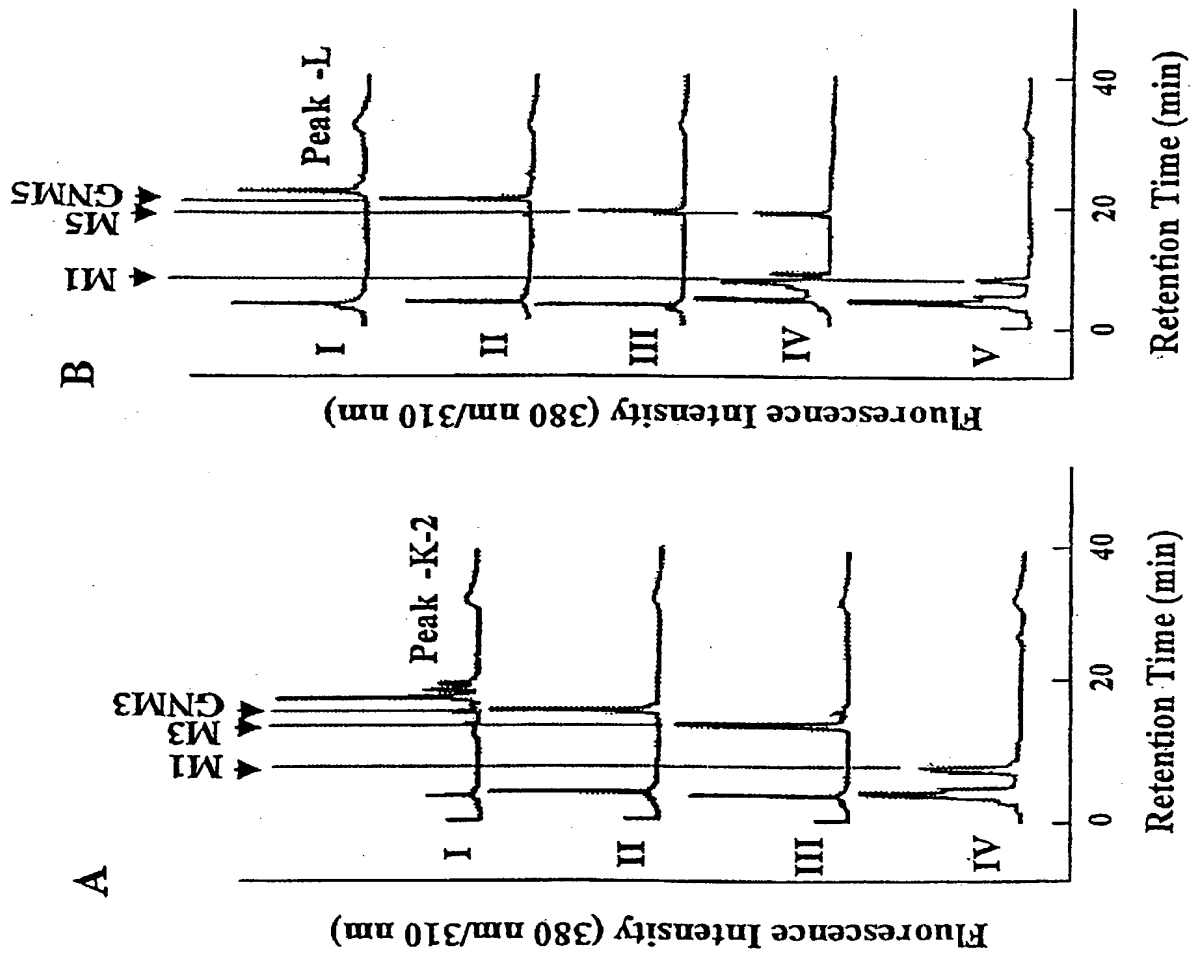


FIG 14

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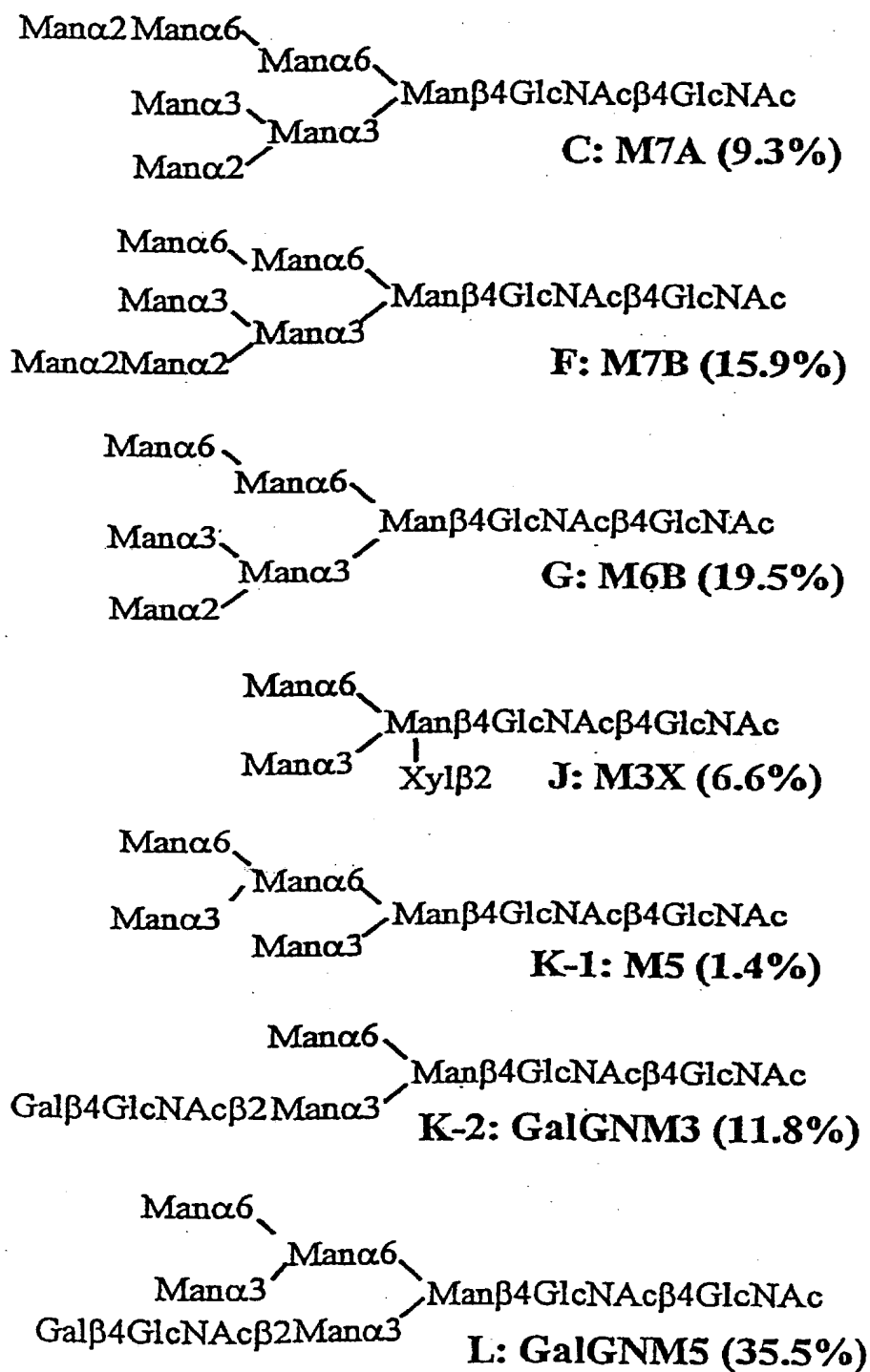


FIG 15

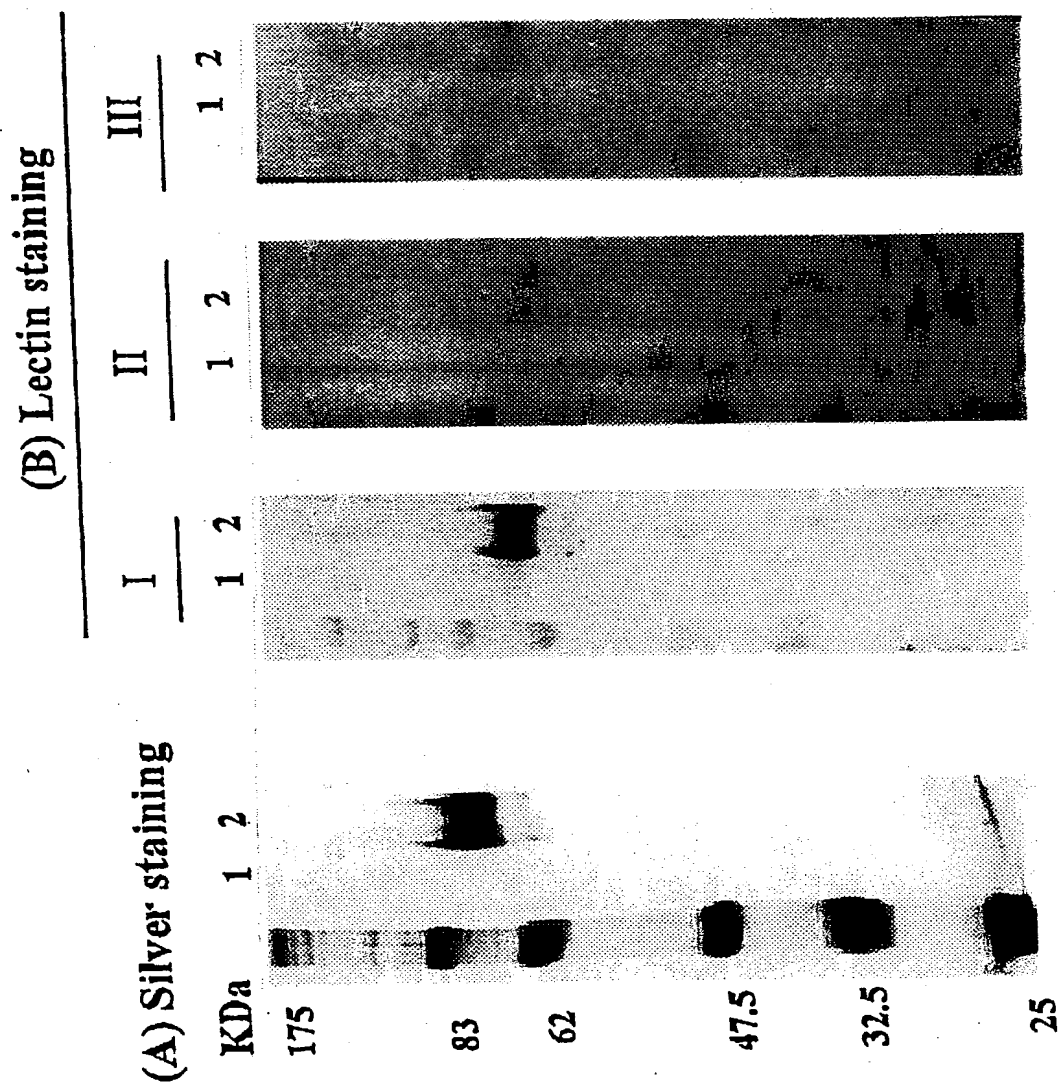


FIG 16

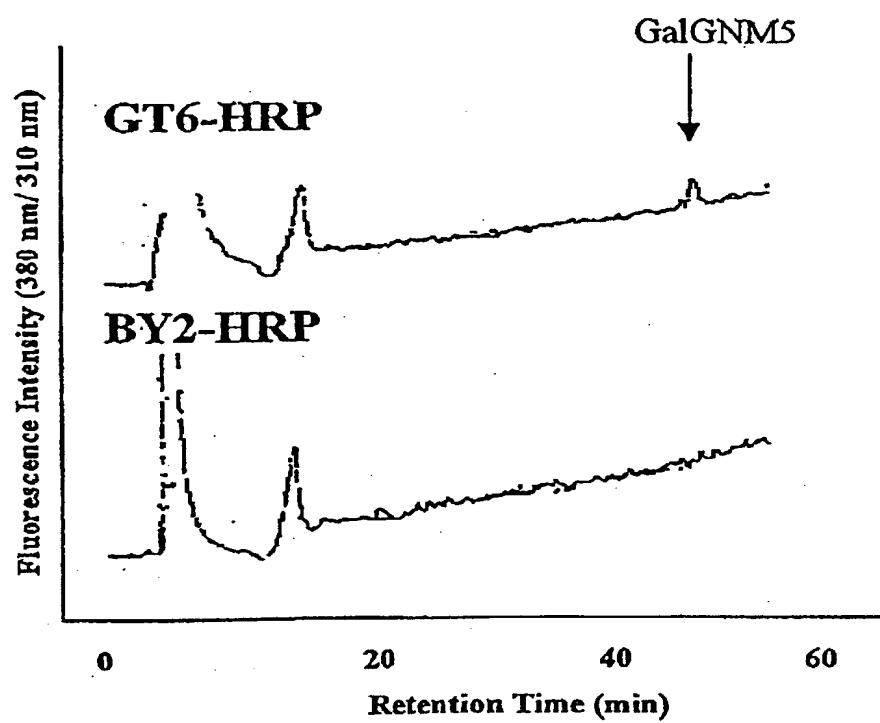


FIG 17

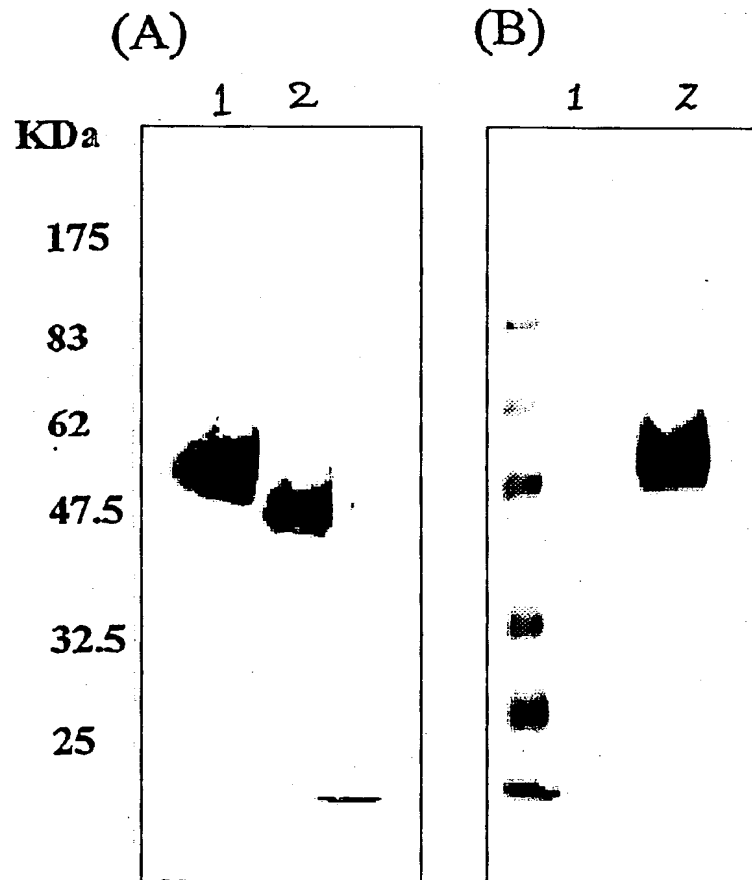


FIG 18

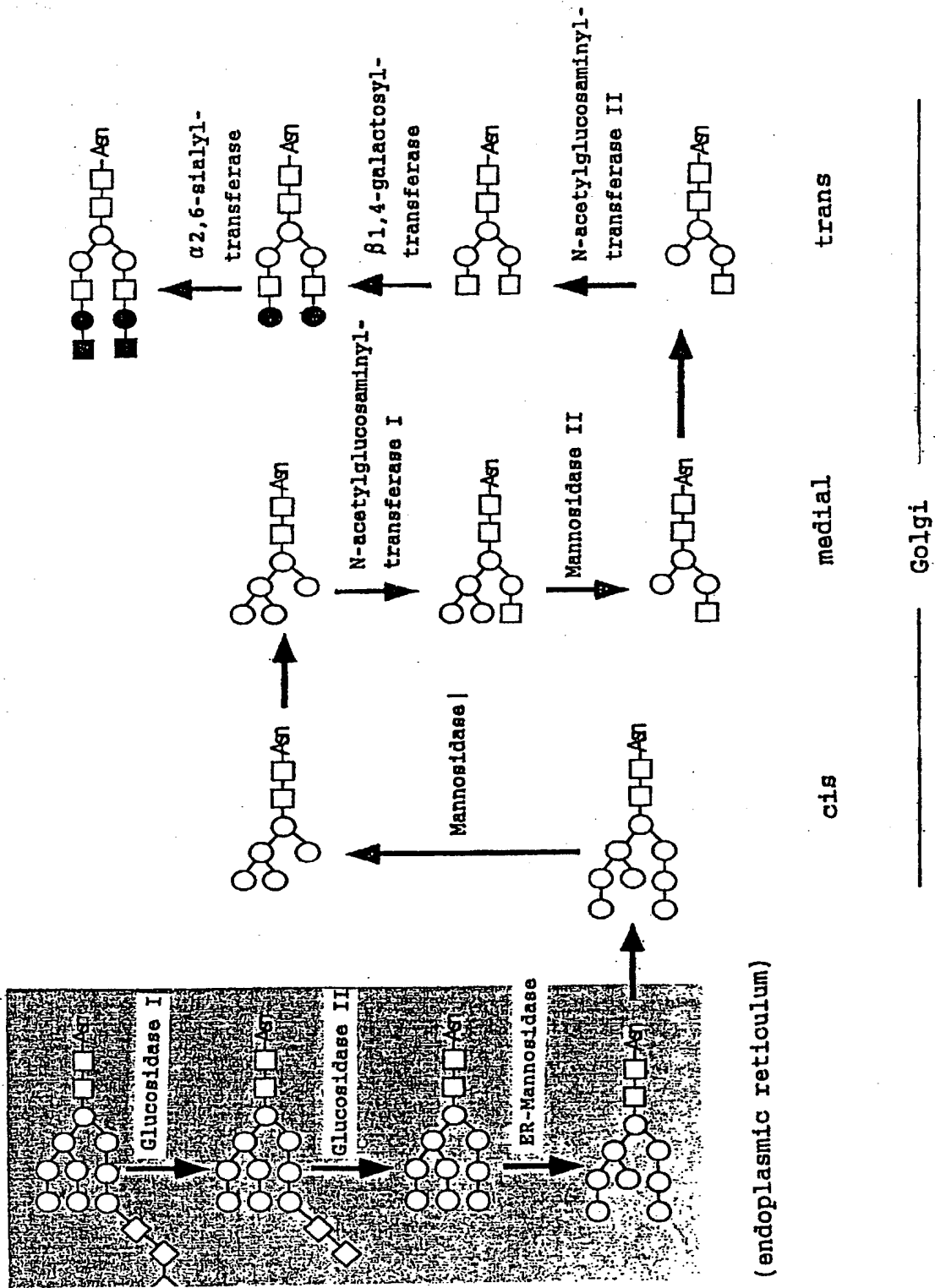
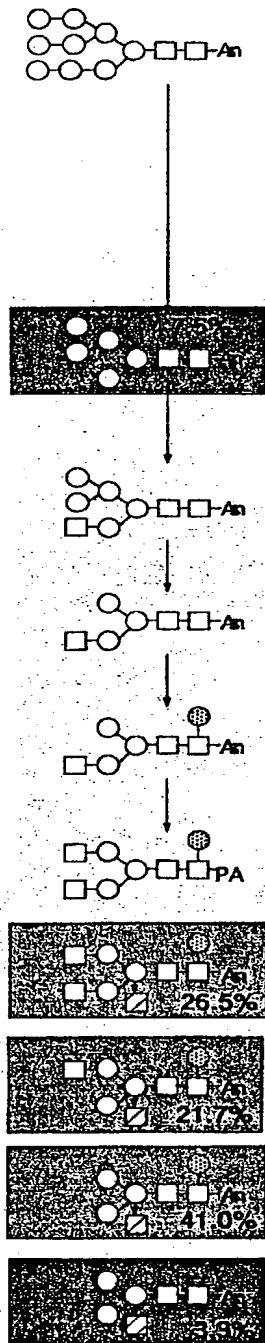


FIG 19

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Wild type; BY2 cell



Transgenic; GT6 cell

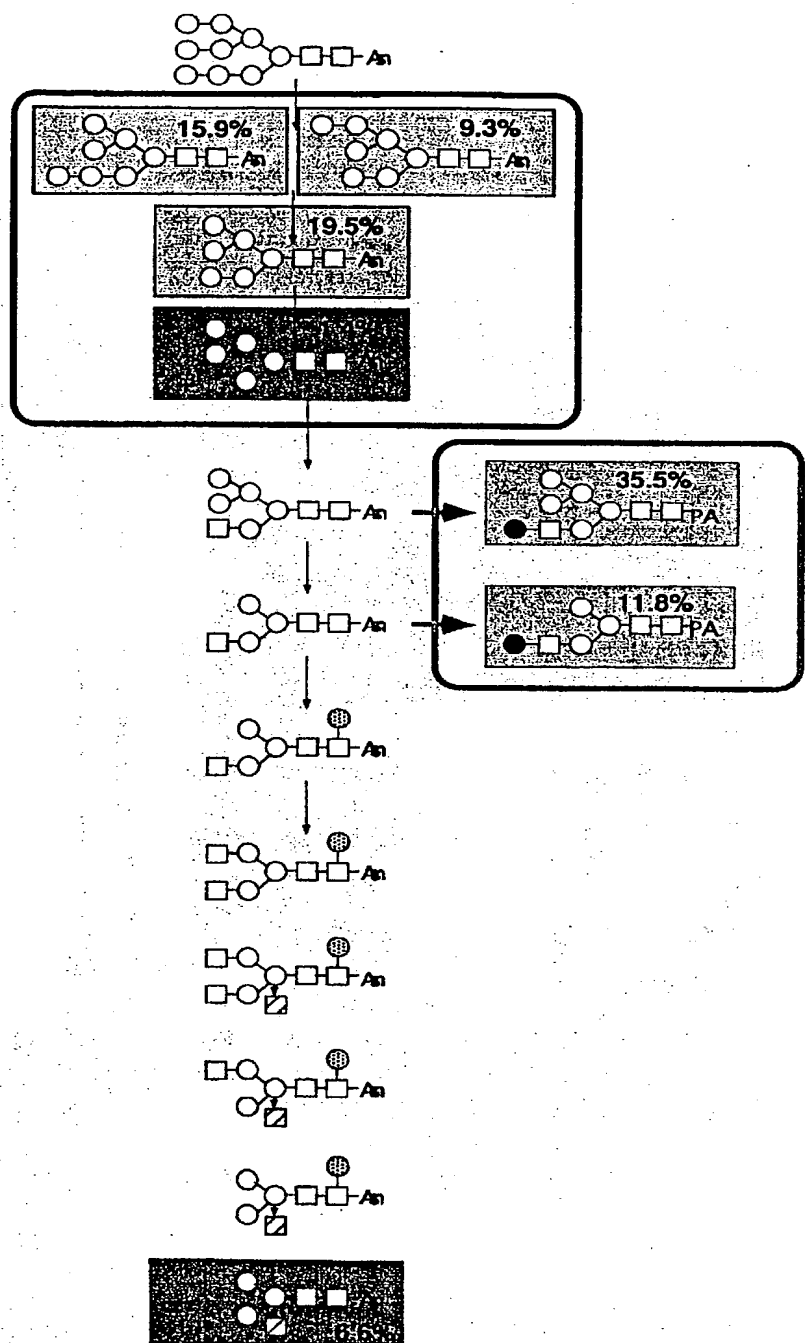


FIG 20

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Super Transgenic GT6 cell

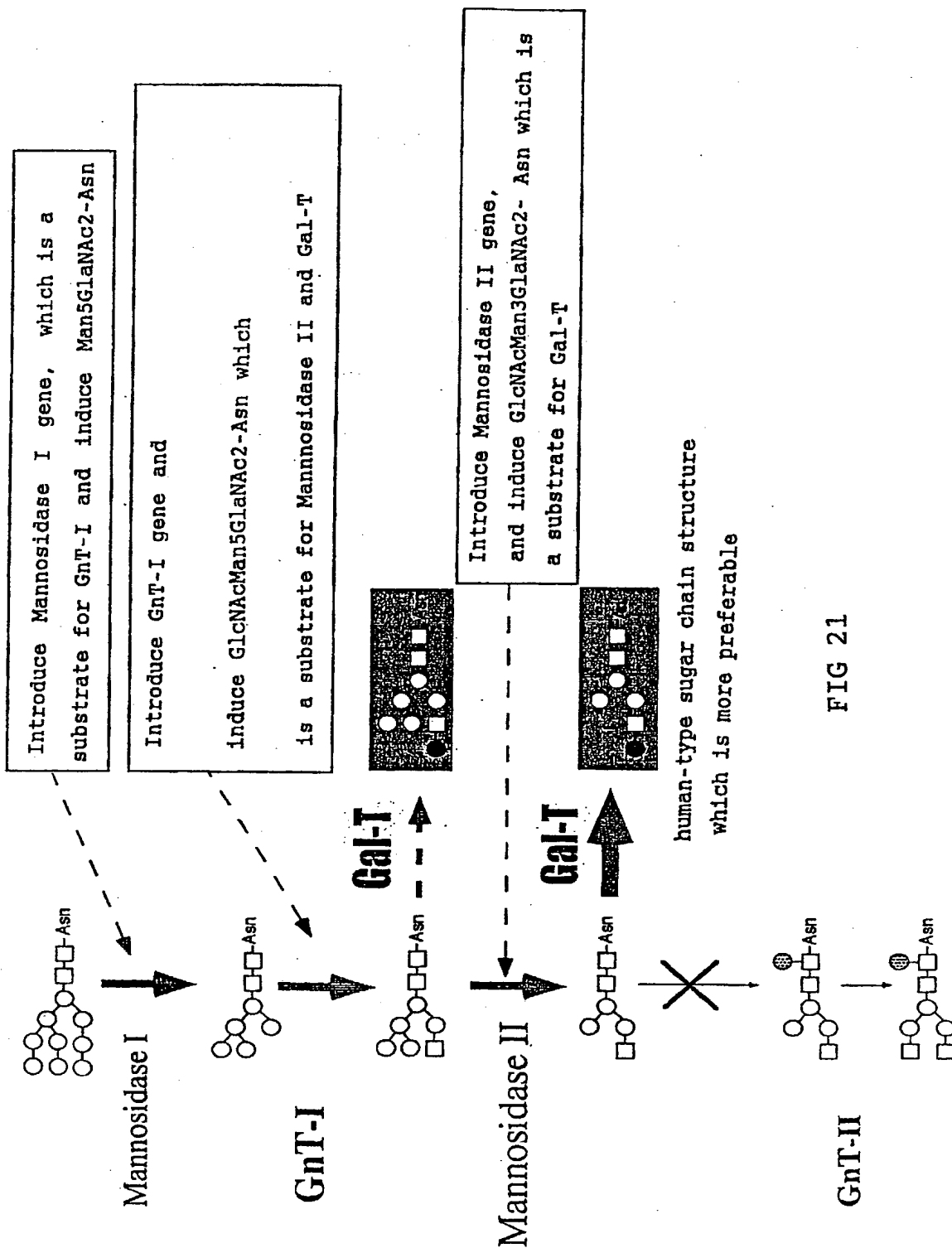
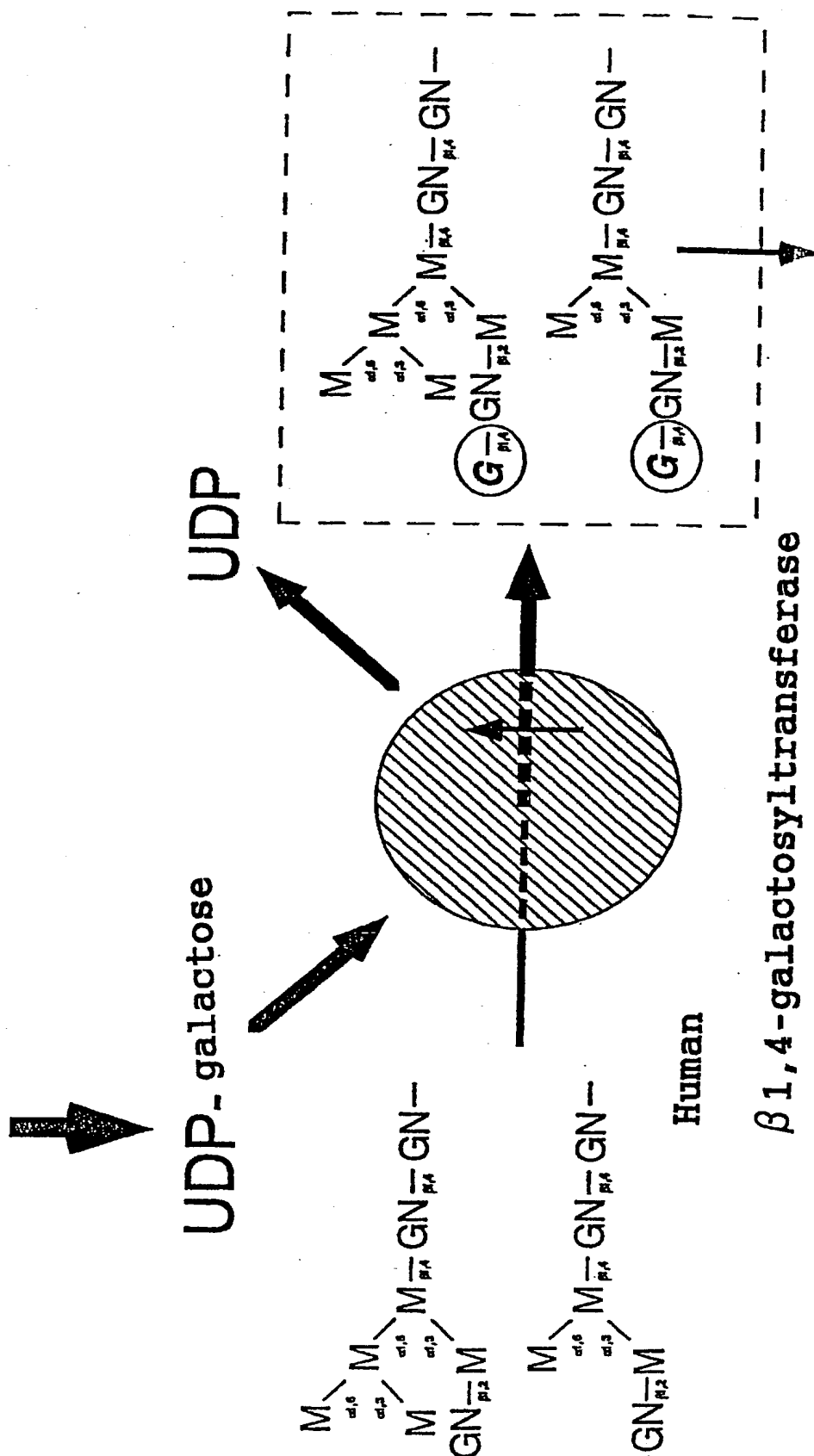


FIG 21

UDP-galactose synthetic enzyme group



Increase of amount of
sugar chains with galactose

FIG 22

β 1,4-galactosyltransferase

Human

09857651 082701

COMBINED DECLARATION AND POWER OF ATTORNEY
(Includes Reference to PCT International Applications)ATTORNEY'S DOCKET NO.
2150-0002-10

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

A METHOD FOR MANUFACTURING GLYCOPROTEINS HAVING HUMAN-TYPE GLYCOSYLATION

the specification of which (check only one item below):

- ☐ is attached hereto.
- ☐ was filed as United States application Serial No. _____ on _____ and was amended on _____, (if applicable)
- ☒ was filed as PCT international application Number PCT/JP99/06881 on 08 December 1999.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56(a).

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate or 365(a) of any PCT international application(s) which designated at least one country other than the United States of America listed below and have also identified below, by checking the box, any foreign application(s) for patent or inventor's certificate, or of any PCT international application(s) having a filing date before that of the application(s) on which priority is claimed.

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COUNTRY (If PCT, indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC §119	
JP	10-350584	09 December 1998	<input checked="" type="checkbox"/> YES	<input type="checkbox"/> NO

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or 365(c) of any PCT international application(s) designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT application(s) in the manner provided by the first paragraph of 35, U.S.C. §112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56(a) which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application.

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U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	PENDING	ABANDONED
PCT APPLICATIONS DESIGNATING THE U.S.				
PCT APPLICATION NO.	PCT FILING DATE	U.S. Serial Nos. Assigned (if any)		
PCT/JP99/06881	08 December 1999		X	

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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 Linda R. Judge 42,702 Larry W. Thrower 47,994

LeeAnn Gortney 37,337
 Jacqie F. Mahoney P48,390

[illegible]

00-1
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00-3

1/8

Sequence Listing

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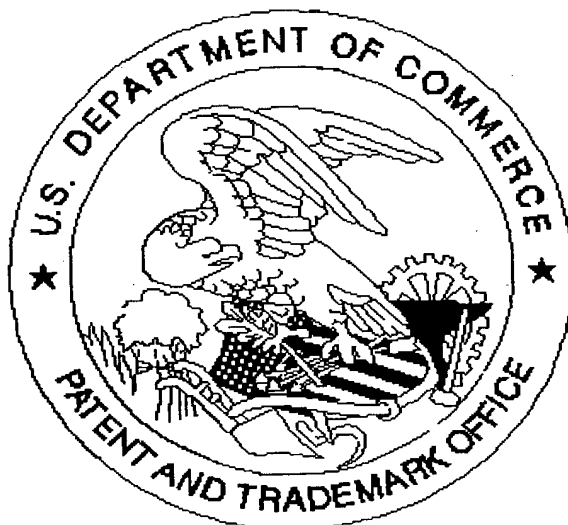
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